Biopanning for *Salmonella* Antigens

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DEDICATION

This thesis is dedicated to my mother, who has never pushed me to change who I am. To my father, whose footsteps I have followed by earning a PhD. To my brother, who made me the nature-loving tomboy that went on to become a scientist. To my older sister, who rooted for me all the way. To my little sister, who put up with my bossiness for so long. To the Power family, who are so close to being my own family. To Marianna Ralli and George Doumanides, who bring so much laughter into my life. To everybody in the lab for listening to my rants. To Dr. Maria Vu, who is the closest thing to a perfect human being and my hero.

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ABBREVIATIONS

AEBSF: 4-(2-aminoethyl)benzenesulfonyl chloride HCl
APC: antigen presenting cell
APS: ammonium persulfate
ATP: adenosine triphosphate
BCIP: 5-bromo-4-chloro-3-indoyl phosphate (p-toluidine salt)
bp: basepairs
BSA: bovine serum albumin
cAMP: cyclic adenosine monophosphate
CRP: catabolite repressor protein
CFTR: cystic fibrosis transmembrane receptor
CFU: colony-forming unit
CLIP: class II-associated invariant chain peptide
CMI: cell-mediated immunity
CTL: cytotoxic T lymphocyte
dATP: deoxyadenosine triphosphate
dCTP: deoxycitidine triphosphate
dGTP: deoxyguanosine triphosphate
dTTP: deoxythymidine triphosphate
DC: dendritic cell
DDA: dimethyl dioctadecyl ammonium bromide
DHFR: dihydrofolate reductase
DMSO: dimethylsulfoxide
DTH: delayed-type hypersensitivity
DTT: dithiothreitol
ELISA: enzyme-linked immunosorbent assay
EDTA: ethylenediamine tetraacetate
ER: endoplasmic reticulum
GFP: green fluorescent protein
GST: glutathione-S-transferase
HRP: horseradish peroxidase
IEC: ion exchange chromatography
IFN: interferon
Ig: immunoglobulin
IL: interleukin
iNOS: inducible nitric oxide synthase
IPAB: iterative panning and binding
IPTG: isopropyl-β-D-thiogalactoside
IVET: *in vivo* expression technology
LB: Luria-Bertani medium
LBP: LPS binding protein
LDS: lithium dodecyl sulfate
LPS: lipopolysaccharide
M cells: membranous epithelial cells
MBP: maltose binding protein
MES: 2-(N-morpholino)ethanesolfonic acid
MHC: major histocompatibility complex

ABBREVIATIONS (continued)

NF-κβ: nuclear factor κβ
Ni-NTA: nickel-nitrilotriacetic acid
NK: natural killer
OD: optical density
ORF: open reading frame
PAI: pathogenicity island
PAMP: pathogen-associated molecular pattern
PBS: phosphate buffered saline
PEG: polyethylene glycol
PFU: plaque-forming unit
pI: isoelectric point
PIPES: Piperazine-N,N'-bis(2-ethanesulfonic acid)
PMN: polymorphonuclear lymphocytes
PP: Peyer's patches
psi: pounds per square inch
PVDF: polyvinylidene fluoride
RT: room temperature
SAS: saturated ammonium sulfate
SCV: *Salmonella*-containing vacuole
SDS: sodium dodecyl sulphate
SDS-PAGE: SDS-polyacrylamide gel electrophoresis
SPI: Salmonella pathogenicity island
STM: signature-tagged mutagenesis
TAE: Tris-acetate-EDTA
TAP: transporter associated with antigen processing
TBE: Tris-borate-EDTA
TEMED: N,N,N',N'-Tetramethylethylenediamine
TLR: Toll-like receptor
TTSS: type three secretion system
WHO: World Health Organisation
w/v: weight per volume

ABSTRACT

Salmonella enterica is a food-borne pathogen that causes substantial morbidity and economic loss worldwide Typhoid fever is the most severe form of salmonellosis and kills 600,000 people annually. The emergence and spread of antibiotic resistance combined with increased international travel places renewed emphasis on effective vaccination strategies. Of the two currently available typhoid fever vaccines, one is potentially inappropriate for immuno-compromised individuals while the other could be rendered ineffective as the pathogen evolves. This work addresses these shortcomings and explores the possibility of identifying novel antigens for use in a multiple subunit vaccine.

S. enterica serovar Typhimurium infection of mice is a well-established animal model of human typhoid fever. Phage display is a method of generating particles that embody a physical link between a given DNA sequence and the polypeptide it encodes. In this work, a phage display library was constructed from random fragments of *S.* Typhimurium genomic DNA. Hyper-immune serum from infected CBA/Ca mice was used for affinity selection of potential antigens from this library in a process called biopanning. Individual clones encoding potential antigens were subjected to further screening alongside a positive and negative control, and 10 were consistently positive. Sequences encoding the potential antigens were then sub-cloned into an expression vector encoding a polyhistidine fusion tag for affinity purification. Six of the ten sub-clones could not be induced to express the fusion peptide, whilst the other four were induced weakly. These remaining four underwent multiple purification steps before being tested by western blot and ELISA alongside the same positive control as used in screening for antigenicity. It was found that the

positive control in itself was poorly antigenic and the four candidates were not antigens, suggesting that other factors interfere with the biopanning and antigen detection process. Approaches for modification of the original affinity selection process and the use of alternative phage-displayed positive control antigens are discussed.

CHAPTER 1

Introduction

A. *Salmonella enterica*

1. General background

Salmonella enterica is a facultative intracellular parasite that is a member of the family *Enterobacteriaceae* and is a close relative of *Escherichia coli*. *S. enterica* is divided into subspecies I-VI, but nearly all the strains that cause disease in humans and domestic animals belong to subspecies enterica (I). These 6 subspecies are composed of over 2000 serotypes (Le Minor, 1988). Serotypes are classified according to Kauffmann-White scheme (also used for typing *E. coli* and other strains) based on antigenic polymorphisms of lipopolysachharide (O) and flagella (H) (Fierer and Guiney, 2001). Serotype names are commonly written as if they were species names (Darwin and Miller, 1999). In this work, serotype names will not be italicised. For example, *S. enterica* serovar Typhimurium will be termed *S.* Typhimurium.

Some serotypes are highly adapted to one host species (such as *S.* Typhi and *S.* Gallinarum in humans and chickens, respectively), while others can cause disease in a variety of hosts (such as *S.* Enteritidis or *S.* Typhimurium). While infections by most serotypes are limited to localised gastrointestinal diseases, some can cause systemic infection and septicaemia, with focal infections such as osteomyelitis and meningitis. These may be associated with, but not limited to, a number of host factors that may predispose the individual to extra-intestinal *Salmonella* infection (Cohen *et al.*, 1987; Wilkins and Roberts, 1988; Darwin and Miller, 1999; Fierer and Guiney, 2001).

2. Non-typhoid salmonellosis in developed & developing countries

The most common form of salmonellosis is a self-limiting diarrhoea, with the majority of these cases resulting from infection by *S.* Typhimurium and *S.* Enteritidis. It is estimated that there are 2 to 4 million cases of infection per year in the United States alone, primarily following the consumption of contaminated beef, poultry, or eggs. *Salmonella* infections are also responsible for substantial economic losses in the livestock industry (Maurice, 1994; Darwin and Miller, 1999).

In the developed world, antibiotic resistance among *Salmonella* strains has arisen primarily through the systematic use of these drugs in food-producing animals for therapeutic, prophylactic, or growth-promoting purposes (Threlfall, 2002). Inappropriate use of antibiotics has led to an increase in virulent, multiply antibiotic-resistant strains of bacteria, such as *S.* Typhimurium DT104. Even more disturbing was the discovery that antibiotic resistance in *S.* Typhimurium DT104 is carried on the chromosome rather than on a plasmid (Threlfall *et al.*, 1994). This strain was first recognised as a problem in the UK, and it was later isolated in countries throughout the world (Glynn *et al.*, 1998; Baggesen *et al.*, 2000; Sameshima *et al.*, 2000). It was also found that this strain continued to become resistant to new drugs (Threlfall *et al.*, 1996) and spread to a wide range of host species (Baggesen *et al.*, 2000).

In the developing world, enteritis caused by non-typhoidal serotypes is more severe and is more frequently associated with invasive illness than enteritis caused by the same or similar serotypes in developed countries. Furthermore, contaminated water is the primary source of disease transmission, rather than consumption of contaminated animal products. Additionally, the appearance of drug resistance is not frequently associated with the use of antimicrobial drugs in livestock, but rather their use in humans (Threlfall, 2002).

3. Typhoid fever

The most severe form of salmonellosis is caused by *S.* Typhi. World wide, it is estimated that over 16 million people develop typhoid fever and 600,000 die annually (Pang *et al.*, 1998). In developed countries, typhoid fever is relatively uncommon, and incidence of this disease is typically associated with travellers who have visited areas of the world where typhoid fever is endemic (Mead *et al.*, 1999). The majority of typhoid fever cases occur in Africa and Asia, with the highest mortality rates in areas where sewage disposal, water purification, and public access to medical care is poor (Jones and Falkow, 1996). The occurrence of antibiotic resistance is also increasing, as manifested by the numerous outbreaks of typhoid fever caused by antibiotic-resistant *S.* Typhi in Asia in the last 15 years (Threlfall, 2002).

Depending on the immune status of the individual and the number of bacteria ingested, the incubation time before initial symptoms are manifested ranges from 5 to 20 days. These symptoms include diarrhoea accompanied by headache, weakness, dizziness, chills, muscle pains, anorexia, and/or abdominal cramping. If the disease is not resolved at this stage, symptoms progress to the typical fever and abdominal pain. Advanced stages of typhoid fever can result in permanent damage to the intestines. Untreated typhoid fever usually resolves after about 4 weeks of infection, although relapses are not uncommon. If treated with antibiotics, the disease usually resolves in 3-5 days. Of those who contract typhoid fever, 1-6% become chronic carriers, as do some who have no clinical history of typhoid (Jones and Falkow, 1996).

4. Animal models of infection

The epidemic of enteritis among humans and livestock caused by *Salmonella* having multiple drug resistance has led to a renewed interest in the study of diarrhoeal salmonellosis. Several animal models have been used to study gastro-enteritis. These include rats, pigs, and rhesus monkeys (Kent *et al.*, 1966; Powell *et al.*, 1971; Reed *et al.*, 1986). However, more recent studies have focused on using calves as the main animal model for this type of disease because of their importance in zoonosis (Tsolis *et al.*, 2000; Santos *et al.*, 2001).

Studying the pathology of human typhoid fever is slightly more complicated than studying enteritis, since *S.* Typhi, the causative agent, is highly adapted to the human host. One animal model of human typhoid fever is the chimpanzee. Oral challenge with 10^{11} virulent *S.* Typhi results in an illness that is similar to, but milder than, human typhoid fever. Since this animal is expensive and not in abundant supply, it is not an ideal model for extensive investigation into the pathogenesis of typhoid fever (Edsall *et al.*, 1960; Pasetti *et al.*, 2003).

By far the most useful animal model has been the mouse. Murine *S.* Typhimurium infection has been a useful, alternative model for studying the disease due to similar pathological characteristics (Carter and Collins, 1974; Jones and Falkow, 1996). However, this model cannot be treated as identical to human infection with *S.* Typhi. Although comparison of the mouse and human genomes showed remarkable similarity between the species, with only about 300 genes not present in both species, it is important to note the differences in size, lifespan, and ecological niches occupied by the two species (Mestas and Hughes, 2004).

Differences are also observed between the immune systems of each species. The difference in size and heart rate between the species substantially affects the rate and distance that an antigen-presenting cell must travel in order to reach lymph nodes. Thus, it is not surprising that the two species exhibit differences in the underlying mechanism and characteristics of the delayed-type hypersensitivity (DTH) response, which is thought to be driven by CD4+ T lymphocyte-mediated activation of macrophages (Mestas and Hughes, 2004). A longer lifespan in humans could be a reason for longer and more diverse T cell memory, as well as the greater presence and redundancy of programmed cell death signalling pathways to avoid tumorigenesis (Chun *et al.*, 2002; Mestas and Hughes, 2004). In addition, human blood contains more neutrophils (50-70%) than lymphocytes (30-50%), compared to mouse blood (75-90% lymphocytes, 10-25% neutrophils) (Doeing *et al.*, 2003). Nevertheless, research on murine infection with *S.* Typhimurium has served as an excellent model for human typhoid fever and has led to a greater understanding of pathogenesis, immunological response, and vaccination against *S.* Typhi (Pasetti *et al.*, 2003). This work will focus on the murine typhoid model, and any incontsistencies with human typhoid fever will be highlighted.

B. *S.* Typhimurium pathogenesis in mice

1. Breaching of the intestinal epithelium

Following oral inoculation of mice with *S.* Typhimurium, bacteria that survive the harsh conditions of the stomach and successfully compete with normal

flora of the small intestine initially colonise the Peyer's Patches (PP) (Bohnhoff and Miller, 1962; Carter and Collins, 1974; Darwin and Miller, 1999). Attachment to the cells of the PP is mediated by long polar fimbriae and enables *S.* Typhimurium colonisation of Peyer's Patches (Baumler *et al.*, 1996). Indeed, multiple fimbrial adhesins are necessary for full virulence of this pathogen in mice (van der Velden *et al.*, 1998). *S.* Typhimurium then breaches the small intestine within a few hours, primarily through the membranous epithelial (M) cells of the PP but also through epithelial cells (Makela and Hormaeche, 1997). M cells allow the immune system to sample antigens from the intestinal lumen by transporting a broad range of materials across the epithelium to underlying lymphocytes and macrophages (Jepson and Clark, 2001).

S. Typhimurium does not passively enter, but rather invades, M cells as well as enterocytes. First, the bacteria attach to the intestinal wall with the aid of multiple fimbrial adhesins. using effector proteins secreted by the type III secrection system (TTSS) of *Salmonella* pathogenicity island I (SPI-1) (Galan and Collmer, 1999). When it comes into contact with M cells, the secreted effector proteins induce membrane ruffling and cytoskeletal rearrangements that result in bacterial internalisation. Multiple *S.* Typhimurium-containing vesicles then fuse to form a larger compartment. This process has cytotoxic effects that lead to host cell destruction, and *Salmonella* can then spread to adjacent cells and underlying phagocytic cells (Jones *et al.*, 1994; Jones and Falkow, 1996).

Although the major site of intestinal breaching is the M cells of PP, it is not surprising that *S.* Typhimurium has the ability to penetrate the intestine by other means, given the fact that M cells comprise about 0.01% of the intestinal

epithelium (Jepson and Clark, 2001). Alternative routes of extra-intestinal dissemination are likely to have a common requirement for intracellular replication, since this is a key determinant of virulence (Leung and Finlay, 1991). One described alternative route of dissemination is via internalisation by dendritic cells (DC) that penetrate the gut epithelium to sample antigens (Rescigno *et al.*, 2001), with subsequent transport to the bloodstream inside CD18+ phagocytes (Vazquez-Torres *et al.*, 1999).

Like murine *S.* Typhimurium infection, human *S.* Typhi infection also manifests characteristic intestinal lesions at sites corresponding to PP (Owen, 1994). However, experimental data show differences in the mechanism by which *S.* Typhi breaches the intestinal epithelium. *S.* Typhi induces the surface expression of cystic fibrosis transmembrane conductance regulator (CFTR) on human intestinal epithelial cells (Lyczak and Pier, 2002). Contact with epithelial cells induces the *S.* Typhi expression of lipopolysaccharide (LPS) core, which is a CFTR ligand (Lyczak *et al.*, 2001). In addition, type IVb pili that mediate bacterial self-association also bind to CFTR and permit host cell invasion mediated by the SPI-1 TTSS (Zhang *et al.*, 2000b; Lyczak *et al.*, 2001; Morris *et al.*, 2003).

Furthermore, commensal bacteria have opposite effects on the ability of *S.* Typhimurium to breach the intestinal epithelium compared to *S.* Typhi. While *S.* Typhimurium must compete with the resident intestinal microflora (Bohnhoff and Miller, 1962), invasion by *S.* Typhi is actually enhanced in their presence. Pre-treatment of epithelial cells with extracts of commensal bacteria increased the surface expression of CFTR by an unknown mechanism. This pre-treatment increased the ability of *S.* Typhi to invade the cells, but had no effect on invasion by *S.* Typhimurium (Lyczak, 2003).

2. Initial host response and *S.* Typhimurium dissemination

The innate immune response is the fundamental host defence against infection that has proven sufficient for the evolutionary survival of most organisms. Only vertebrates have an additional, adaptive response that uses immunological memory to protect the host from future infections by specific pathogens. Still, adaptive immunity cannot protect the host unless the innate immune response controls infection during the time required to develop the pathogen-specific response (Beutler, 2004).

Upon invasion of host cells in the intestine by *S.* Typhimurium, polymorphonuclear lymphocytes (PMN) are the first immune cells to accumulate in the sub-mucosa, in response to the production of chemokines by the infected intestinal cells. The rapid appearance of PMN at sites of infection is an innate immune response that is conserved among all host species that have been studied (Fierer, 2001). Neutrophils, a type of PMN, are responsible for controlling bacterial numbers at the site of intestinal breach (Cheminay *et al.*, 2004). Neutrophils and macrophages, which are critical to the survival of *Salmonella*-infected mice, share a common microbicidal role in the innate immune response (Mittrucker and Kaufmann, 2000). These phagocytes kill bacteria with the aid of toxic reactive oxygen and nitrogen intermediates that are generated by a series of reactions initiated by NADPH oxidase (Beutler, 2004). Mouse macrophages also produce inducible nitric oxide synthase (iNOS), which is central to the elimination of *S.* Typhimurium (Alam *et al.*, 2002). However, whether or not human macrophages make iNOS remains controversial (Mestas and Hughes, 2004).

Upon breaching the intestinal epithelium, a proportion of *Salmonella* gain access to the blood. During this brief extracellular state, the pathogen becomes

susceptible to attack by the host complement system (See section D1b). Although the intracellular state of *Salmonella* protects it from complement during most of the infection, murine complement deficiency increases susceptibility to *S.* Typhimurium infection (Warren *et al.*, 2002). As part of the defence strategy, *S.* Typhimurium produces a 17 kDa outer membrane protein that mediates protection from complement by interfering with the formation of a fully polymerised tubular membrane attack complex. This protein is encoded by the *rck* gene carried on the pSLT virulence plasmid (Heffernan *et al.*, 1992). On the contrary, *S.* Typhi is shielded from complement killing in non-immune mice by producing an exopolysaccharide (Robbins and Robbins, 1984).

Salmonella that escape rapid clearance from the circulation subsequently reach the reticuloendothelial system (RES), where they mainly reside in PMN, macrophages, and dendritic cells. At the primary sites of extra-intestinal colonisation, *S.* Typhimurium is mainly associated with resident Kupffer cells of the liver, and the red pulp macrophages and marginal zone macrophages of the spleen. At these sites, bacteria continue to replicate inside macrophages and to infect other macrophages. This eventually leads to the formation of a focal lesion. As the infection progresses, the number of lesions increases as the host attempts to prevent the uncontrolled spread of *Salmonella* throughout the body (Mastroeni and Sheppard, 2004). At the cellular level, many complex interactions between macrophage and *S.* Typhimurium that depend on bacterial and host factors determine the course of the infection.

C. Horizontally acquired genes conferring *Salmonella* virulence

1. Overview

Over the last several years, a host of genes that contribute to *S.* Typhimurium pathogenesis have been identified. Many encode proteins involved in basic metabolic pathways, gene regulation, stress responses, and defence against host antimicrobial factors. Many, but not all, have been shown to be vital for full virulence (Hoiseth and Stocker, 1981; Hensel *et al.*, 1995; Bowe *et al.*, 1998; Taylor *et al.*, 1998; Garcia-Del Portillo *et al.*, 1999; Humphreys *et al.*, 1999; Lundberg *et al.*, 1999a).

A significant fraction of virulence genes are the product of horizontal gene transfer. These include adhesins, invasins, toxins, and other factors that have been added to the chromosome through conjugation, transformation, and transduction. This lateral transfer enables *Salmonella* to expand its host range and is considered the most efficient mechanism of bacterial evolution (Ochman *et al.*, 2000). Indeed, the level of genetic variability is much less pronounced in host-restricted *Salmonella* serotypes, such as *S.* Typhi (Selander *et al.*, 1990).

Clusters of virulence genes that have become integrated in the chromosome are called pathogenicity islands (PAI). About 75% of PAI are associated with tRNA genes, which are frequently used as integration anchor points by temperate bacteriophages (Hou, 1999). This is also true in the case of *S. enterica*, with all but one of the known PAI being flanked by a tRNA gene (Hansen-Wester and Hensel, 2002). In fact, most of the tRNA-encoding loci have been used as anchor points for lateral gene transfer during *Salmonella* evolution (Porwollik and McClelland, 2003). Since many characteristics of *Salmonella* virulence are the phenotypic result of

horizontally acquired genes, the following section will focus on these loci and their regulation in pathogenesis.

2. *Salmonella* Pathogenicity Islands (SPI)

The *E. coli* K12 genome is approximately colinear with that of *S.* Typhimurium. *Salmonella* pathogenicity islands (SPI) contain clusters of genes that are absent from the corresponding location in the *E. coli* chromosome (Fierer and Guiney, 2001). *S. enterica* harbours several SPI that enable it to invade its host and proliferate. SPI-1 through SPI-5 have been partially characterised in *S.* Typhimurium, although SPI-1 and SPI-2 have been the focus of most studies. SPI-4 (25 kb) contains a gene required for survival in macrophages as well as putative toxin secretion genes. SPI-5 is a 7 kb region with a composite nature and encodes effector proteins secreted by the TTSS of SPI-1 as well as SPI-2 (Schmidt and Hensel, 2004). The presence of other SPI has been inferred from the complete genomic sequence of *S.* Typhimurium (McClelland *et al.*, 2001).

SPI-3 is a 17 kb region that harbours a high-affinity magnesium transport system that is important for survival inside macrophages. The *mgtC* gene, which is required for growth in low Mg^{2+} concentrations, has no homologue in *E. coli*. However, the macrophage-tropic intracellular pathogen *Mycobacterium tuberculosis* harbours an *mgtC* homologue that permits growth in low Mg^{2+} concentrations and that is required for intracellular proliferation and virulence in mice (Buchmeier *et al.*, 2000). Since *E. coli* cannot grow in Mg^{2+} concentrations as low as *S.* Typhimurium, the horizontal acquisition of this element may have been an important step in the evolution of *Salmonella* as a pathogen (Blanc-Potard and Groisman, 1997).

S. enterica is unusual among bacteria in that it carries two virulence-associated TTSS. All *Salmonellae* harbour the SPI-1 TTSS, although the repertoire of effector proteins secreted by this system is not the same for all serotypes. The SPI-2 TTSS, which is present in all *S. enterica* serovars, is absent from *S. bongori*. *S. bongori* is a commensal organism of cold-blooded vertebrates that is rarely associated with human infection. It is believed that the two pathogenicity islands were acquired as independent events, and that SPI-2 was acquired after SPI-1. Furthermore, SPI acquisition probably also took place in multiple events, since each SPI contains a segment of genes with a base composition that differs from the rest of the SPI and that is not required for virulence (Reeves *et al.*, 1989; Boyd *et al.*, 1996; Ochman and Groisman, 1996; Hansen-Wester and Hensel, 2001).

SPI-1 comprises a region of approximately 40kb on the chromosome. The SPI-1 TTSS is known to secrete at least 19 polypeptides; however, the genes encoding the majority of effector proteins are located elsewhere on the chromosome, in association with other pathogenicity islands and mobile genetic elements (Galan, 2001; Hansen-Wester and Hensel, 2001). SPI-1 is critical for *S.* Typhimurium entry into cultured murine M cells and promotes the invasion of M cells in vivo. This is achieved by the injection of effector proteins into the host cell through a molecular syringe (Kimbrough and Miller, 2002). Orally administered mutant bacteria lacking SPI-1 maintain a significant ability to colonise PP, to disseminate outside the intestine, and to exhibit some virulence, although to a significantly lesser degree. On the contrary, SPI-1 deficient bacteria were equally virulent to the wild-type control when injected intraperitoneally (Galan and Curtiss, 1989; Penheiter *et al.*, 1997; Clark *et al.*, 1998; Jepson and Clark, 1998; Vazquez-Torres *et al.*, 1999). It is not surprising that a degree of virulence is retained without functional SPI-1 TTSS in

orally administered bacteria, since it is known that *Salmonella* can disseminate by alternate means (Vazquez-Torres *et al.*, 1999; Rescigno *et al.*, 2001).

SPI-2 is a 40 kb locus that contains 44 *Salmonella*-specific open reading frames. The total number of effector proteins translocated by SPI-2 is unknown, and the functions of those already identified are only partially understood (Hansen-Wester and Hensel, 2001; Hansen-Wester *et al.*, 2004). In contrast to SPI-1, SPI-2 is essential for virulence in mice, regardless of the inoculation route (Ochman *et al.*, 1996; Shea *et al.*, 1996). This has led to the widely accepted conclusion that SPI-2 is required for systemic infection (Hensel, 2000). Expression of the SPI-2 TTSS occurs only after invasion of host cells and subsequent acidification of the *Salmonella*-containing vacuole (SCV) (Cirillo *et al.*, 1998; Beuzon *et al.*, 1999; Pfeifer *et al.*, 1999; Nikolaus *et al.*, 2001). SPI-2 allows *Salmonella* replication in non-phagocytic cells and survival in macrophages. General vesicular trafficking in host cells and maintenance of the SCV are affected by the SPI-2 encoded SpiC and SifA proteins, respectively (Uchiya *et al.*, 1999; Beuzon *et al.*, 2000; Guy *et al.*, 2000). SPI-2 also interferes with the host cell's antimicrobial defences involving reactive oxygen and nitrogen intermediates (Vazquez-Torres *et al.*, 2000; Chakravortty *et al.*, 2002).

SPI-1 and SPI-2 are activated by different environmental stimuli. In the gut lumen, SPI-1 expression is activated by low oxygen levels and high osmolarity. Inside host cells, SPI-2 expression is activated by low Mg^{2+} levels and phosphate starvation (Hansen-Wester and Hensel, 2001). The inability to achieve simultaneous induction of both systems indicates the possibility that they may be inversely regulated (Deiwick *et al.*, 1999). In addition to environmental components, SPI expression depends on the bacterial growth phase. For example, transient expression of SPI-1 was responsible for a striking ability to induce rapid apoptosis in cultured macrophages. This was triggered by prior growth of the bacteria to a very narrow,

specific point of growth, at the transition from exponential to stationary phase (Lundberg *et al.*, 1999b). SPI-1 and SPI-2 have distinct functions in pathogenesis; however, the sequential expression of these two systems raises the possibility that a temporal coordination mechanism may exist. Indeed, it has been shown that mutation of some SPI-2 genes can influence the expression of SPI-1 genes (Deiwick *et al.*, 1998).

A pathogenicity island present in *S.* Typhi, *S.* Dublin, and *S.* Paratyphi C has been identified that is not found in other *Salmonella* strains (Pickard *et al.*, 2003). Unlike SPI-1 and SPI-2, SPI-7 (also called Major PAI) is unstable and can excise spontaneously from an *S.* Typhi clinical isolate when grown in the laboratory (Bueno *et al.*, 2004). Furthermore, clinical *S.* Typhi isolates missing this region have also been characterised (Nair *et al.*, 2004). In *S.* Typhi, this 134 kb pathogenicity island includes the *viaB* operon for the biosynthesis and export of the Vi exopolysaccharide, the *pil* operon for production of type IVb pili, and *sopE*, a bacteriophage-associated effector protein of the SPI-1 TTSS (Pickard *et al.*, 2003). Since Vi-positive *S.* Typhi strains (comprising the majority of clinical isolates) are more virulent than Vi-negative strains (Hornick *et al.*, 1970; Robbins and Robbins, 1984), acquisition of SPI-7 is likely to have been an important step in the evolution of this pathogen.

3. The *spv* operon

A third cluster of virulence genes is carried on the frequently self-transmissable 90 kb pSLT virulence plasmid of *S.* Typhimurium (Ahmer *et al.*, 1999). All strains of *Salmonella* that exhibit virulence in mice carry a virulence plasmid encoding the *spv* operon. This highly conserved operon is the plasmid-borne

component responsible for the murine systemic infection phenotype (Guiney *et al.*, 1995).

As with proteins secreted by the SPI-2 TTSS, *spv* proteins are expressed in the late logarithmic to stationary phase of bacterial growth, induce morphological changes in host cells, and allow intracellular proliferation of *Salmonella*. Expression of *spv* genes is controlled by the stationary-phase sigma factor RpoS (Chen *et al.*, 1995). Within the operon, the product of *spvR* regulates the expession of the four structural components encoded by *spvA*, *spvB*, *spvC*, *spvD* (Guiney *et al.*, 1995). Mutation of *spvR* effectively abolishes expression of the other four genes. The *spvB* and *spvC* genes are sufficient for plasmid-borne virulence and can replace the function of the entire *spv* operon when bacteria are inoculated sub-cutaneously, but not orally, into mice (Matsui *et al.*, 2001). Like SPI-2, *spv* is essential for *Salmonella*-induced apoptosis of intestinal epithelial cells in tissue culture (Paesold *et al.*, 2002). However, experimental evidence shows that the two sets of genes operate by different mechanisms. SpvB, which catalyzes the ADP-ribosylation of actin and is essential for murine virulence, is secreted by a non-TTSS pathway into host cells and induces apoptosis in eukaryotic cells (Gotoh *et al.*, 2003; Kurita *et al.*, 2003). Furthermore, *in vivo* experimental evidence shows that SPI-2 and *spv* contribute independently to murine *Salmonella* virulence (Shea *et al.*, 1999).

Epidemiological evidence supports a role for *spv* in extra-intestinal dissemination of *S.* Typhimurium in humans (Fierer *et al.*, 1992). In fact, the *spv* locus is found among a wide range of *Salmonella* serovars, although in many cases it has become integrated in the chromosome. However, the absence of *spv* genes in the *S.* Typhi genome suggests that the basis of typhoid fever pathogenesis is fundamentally different from that of systemic infection by non-typhoid *Salmonellae* (Guiney *et al.*, 1995; Boyd and Hartl, 1998).

4. Bacteriophage-associated virulence genes

Several functional phage genomes are present in *Salmonella*, with some found only in specific strains. For example, *S.* Typhimurium strain LT2 releases Fels-1, Fels-2, Gifsy-1, and Gifsy-2 phage particles in response to DNA stress. Other *S.* Typhimurium strains do not carry Fels-1; however, they are known to carry Gifsy-3 and SopEϕ. Cryptic prophages and phage remnants are also found throughout the *Salmonella* serovars (Porwollik and McClelland, 2003). Among these bacteriophage-associated sequences lie genes encoding key factors that affect pathogenesis.

The *Salmonella*-specific P22 phage is known to carry genes that modify its surface receptor, LPS. Since a considerable proportion of anti-*Salmonella* antibodies are directed against LPS, O-antigen modification is one of the most important means of immune escape by *Salmonella.* LPS modification may be a P22 mechanism of preventing super-infection by other phages, but changes in the O-antigen also render host antibodies to the previous, unmodified form useless. However, other chromosomal genes are also responsible for O-antigen variation (Fierer and Guiney, 2001). Furthermore, there is no evidence that LPS modification is required for virulence, although there is an example of increased murine *S.* Choleraesuis virulence associated with phage 14 lysogeny-induced O-antigen modification (Nnalue *et al.*, 1990).

The Gifsy-2 phage carries several genes that are directly involved in the pathogenesis process. The *sseI* gene encodes a TTSS effector protein involved in *Salmonella*-induced actin polymerisation in host cells (Miao *et al.*, 2003). Interestingly, Gifsy-2 also carries an antivirulence component whose mechanistic role in infection has yet to be elucidated (Ho and Slauch, 2001). However, the most relevant Gifsy-2 genes in pathogenesis are *gtgE* and *sodCI.* These two genes are

responsible for the observed 100-fold attenuation of *S.* Typhimurium when it is cured of Gifsy-2. GtgE is a protein of unknown function that is produced in laboratory conditions as well as *in vivo* (Ho *et al.*, 2002).

SodCI is a well-characterised periplasmic Cu^{2+}, Zn^{2+} superoxide dismutase with one of the highest catalytic rates ever observed for a superoxide dismutase (Pesce *et al.*, 2000). This enzyme catalyses the conversion of two superoxide anions to one molecule of hydrogen peroxide and one molecule of oxygen. Its periplasmic location helps to protect the cell against exogenous superoxide, thus enhancing the ability to survive the effects of NADPH oxidase inside phagocytes (Fang *et al.*, 1999). SodCI is only present in some of the most pathogenic serotypes, with the notable exceptions of *S.* Paratyphi and *S.* Typhi (Fang *et al.*, 1999). Although the contribution of *sodCI* to virulence is well established, its exact role in the context of *grvA*, the Fels-1 encoded *sodCIII*, or the chromosomally encoded *sodCII* remains unclear (Boyd and Brussow, 2002; Janssen *et al.*, 2003).

The Gifsy-1 phage carries a gene *gipA* that is specifically induced in *S.* Typhimurium when it reaches the small intestine of mice. This protein is important for growth and colonisation in the PP (Stanley *et al.*, 2000). Gifsy-1 also carries a leucine rich TTSS effector protein encoded by *gogB*. Although Gifsy-1 is less important to *S.* Typhimurium virulence than Gifsy-2, its contribution becomes apparent when all Gifsy-2 elements except *sodCI* are removed. It has been suggested that Gifsy-1 contains one or more virulence determinants whose function(s) overlap with element(s) encoded by Gifsy-2 (Figueroa-Bossi and Bossi, 1999).

Gifsy-3 was identified as a novel phage present in *S.* Typhimurium strain ATCC14028 that carries two genes (*pagJ* and *sspH1*) associated with pathogenesis (Figueroa-Bossi *et al.*, 2001). Inactivation of the PhoP/PhoQ-activated *pagJ* locus is known to reduce virulence 1000 fold (Belden and Miller, 1994). The leucine rich

SPI-1 TTSS effector protein encoded by *sspH1* is translocated to the mammalian nucleus and interferes with gene expression, which ultimately leads to a down-regulation of the inflammatory response (Haraga and Miller, 2003). Still, curing ATCC14028 of Gifsy-3 had no detectable effect on virulence in mice (Figueroa-Bossi *et al.*, 2001). This may have been due to a redundancy of these genes in *Salmonella* (Miao *et al.*, 1999; Amavisit *et al.*, 2003).

The SopEϕ bacteriophage was originally identified in *S.* Typhimurium SL1344 when a gene encoding a SPI-1 TTSS effector protein, *sopE*, was found to be located within its genome (Hardt *et al.*, 1998b). SopE is an activator of host cell Rho GTPases, and translocation into the cell leads to the membrane ruffling that induces macropinocytosis of *Salmonella* (Hardt *et al.*, 1998a). A homologue of SopE (known as SopE2) that activates a different set of host cell Rho GTPases is well conserved among *Salmonella* serotypes (Prager *et al.*, 2000; Ehrbar *et al.*, 2002). The SopEϕ-encoded *sopE* gene, however, is found in only a few serotypes associated with epidemics (Mirold *et al.*, 1999). Further evidence to support a role for *sopE* in virulence is its characteristic presence in *S.* Paratyphi B isolates from systemic infections and absence in isolates from enteric infections (Prager *et al.*, 2003).

When analysing the transfer of many phage-encoded virulence genes between strains, a common theme emerges. Many of these genes, including *sseI*, *gogB*, *pagJ*, *sspHI*, and *sopE*, are located within the tail-fibre region of the bacteriophage genomes (Boyd and Brussow, 2002). Selective pressure apparently leads to more rapid evolution of tail fibre genes than other phage genes (Haggard-Ljungquist *et al.*, 1992). Thus, there appears to be an inherent genetic flexibility in this region. Since phage attach to host receptors via tail fibres or tailspike proteins, variability in the genes that encode them can enable phage to modify their host specificity. This can create a new niche or a possible selective advantage for future generations of phage

as they evolve. Furthermore, if the variation takes the form of accessory genes (called morons) such as those listed above, the evolutionary fitness of the host is potentially enhanced. This would provide the host with a disincentive for removing the lysogenised phage, which in turn would give an evolutionary advantage to phage that carry morons (Hendrix *et al.*, 2003). Finally, stresses experienced by a bacterial pathogen during the course of infection may induce otherwise dormant prophages. Indeed, live phage particles are known to be produced in the course of some bacterial infections. Furthermore, structural components of phage particles can be directly pathogenic (Waldor, 1998). The revelation that exposure of *S.* Typhimurium to H_2O_2 induces the release of fully functional Gifsy-1 and Gifsy-2 prophages raises the possibility that *in vivo* prophage induction plays a role in virulence (Figueroa-Bossi and Bossi, 1999).

D. Regulation of *Salmonella* gene expression in the host

1. Overview

Virulence genes are undoubtedly crucial to pathogenesis; however, they must be expressed at the appropriate stage of infection in order to function properly. A recent study identifying candidate antigens for subunit vaccination against *Salmonella* highlighted the importance of gene regulation in vivo for the selection of appropriate antigens (Rollenhagen *et al.*, 2004). *Salmonella*, like other organisms, senses and adapts to environmental conditions by regulating the expression of its genes at many levels. Such conditions include temperature, osmolarity, nutrient availability, pH, gravity, growth phase, and the concentration of oxygen, specific ions, peptides, and other small molecules. Regulation may occur at the level of

transcription, mRNA stability, translation, or post-translational modification. Disruption of the ability to regulate this expression can interfere with pathogenesis. Indeed, numerous regulatory components are known to be essential for virulence (Clements *et al.*, 2001).

2. Control of sequential events during the course of invasion

a. *S.* Typhimurium

S. Typhimurium has a type III flagellar protein export apparatus which secretes peritrichous flagella providing motility. While the expression of flagella during the course of infection is proven by the production of host antibodies to H antigen, the precise role of flagella in virulence remains unclear. For example, interfering with the ability of *S.* Typhimurium to switch between phase I and phase II flagellin at the level of regulation resulted in decreased virulence in the murine typhoid model, but had no effect on bovine gastroenteritis (Ikeda *et al.*, 2001). On the other hand, abrogation of the ability to produce flagella in the first place did not diminish *S.* Typhimurium virulence in mice but had a negative effect on its capacity to invade intestinal epithelial cells and to cause fluid accumulation in the ligated calf intestine (Lockman and Curtiss, 1990; Ikeda *et al.*, 2001; Schmitt *et al.*, 2001).

Nearly 50 genes encoding the proteins responsible for flagellar biosynthesis and function are located in 17 operons on the *S.* Typhimurium chromosome (Kutsukake *et al.*, 1988). Production of flagella is influenced by environmental stimuli that include oxygen and nutrient availability, pH, temperature, and osmolarity. Since motility is energetically expensive to the cell, it is not surprising that the synthesis of flagella is regulated by a complex network. This network

includes a hierarchical organisation of flagellar genes into three classes: 1) genes encoding FlhD and FlhC, which form a heterotetramer that activates class 2) genes encoding hook-basal body complexes and an alternative sigma factor FliA (RpoF) that activates transcription of class 3) genes encoding the filament protein as well as the hook-associated, motor, and chemotaxis proteins. Additionally, the expression of these genes is influenced by other transcriptional activators and repressors, and by mRNA stability through binding of the CsrA global regulator (Lawhon *et al.*, 2003; Soutourina and Bertin, 2003; Teplitski *et al.*, 2003).

New regulators continue to be discovered in *Salmonella*, such as RtsA and RtsB. Interestingly, these are absent in *E. coli* and appear to be part of a horizontal acquisition event. RtsB binds to the *flhDC* promoter and represses the production of flagella; RtsA activates the transcription of *hilA*, which in turn up-regulates the expression of SPI-1 genes (Ellermeier and Slauch, 2003). Together with BarA/SirA and *csrB*/CsrA, RtsB and RtsA are involved in the coordinated regulation of motility and invasion as *Salmonella* passes from the extracellular to the intracellular state. Further experimentation will reveal how these and perhaps other components work together during the early stages of infection.

b. *S.* Typhi

In contrast to *S.* Typhimurium, *S.* Typhi invasion of epithelial cells requires intrinsic, intact motility, and most strains produce only one phase of flagellin (Liu *et al.*, 1988; Arricau *et al.*, 1998). Differences in the mechanism of invasion between the two serotypes may be related to the response to environmental stimuli. For example, environmental osmolarity seems to have a stronger effect on SPI-1 protein secretion in *S.* Typhi than in *S.* Typhimurium. Additionally, serotype-unique

elements may also play a role, since production of flagella and secretion of SPI-1 proteins in *S.* Typhi is in part post-translationally controlled by the presence of the Vi polysaccharide capsule (Arricau *et al.*, 1998).

As described at the end of section B1, *S.* Typhi attachment to host cells is mediated by interaction of LPS core and type IV pili with surface-expressed CFTR. *S.* Typhi produces type IV pili that mediate bacterial self-association, as well as type V pili that inhibit self-association. At the end of the *pil* operon is a segment of DNA flanked by a pair of 19-bp inverted repeats (a simple shufflon), followed by the *rci* gene encoding a shufflon-specific recombinase. Due to DNA inversion mediated by Rci, the *pilV* gene encodes two products with different C-terminal amino acid sequences. However, Rci activity physically interferes with transcription of the *pilV* gene, which indicates that Rci activity and *pilV* expression are inversely correlated. Since Rci activity is influenced by the extent of DNA supercoiling, environmental conditions that promote supercoiling (such as low levels of oxygen in the gut) may lead to decreased expression of *pilV* (Morris *et al.*, 2003). Under these circumstances, the formation of bacterial aggregates may enhance uptake by host cells and may help protect individual cells from host defences. Strains of *S.* Paratyphi C (which can cause typhoid fever in humans) that are Vi^+ also carry the equivalent *pil* operon, but with an important difference: the shufflon is rendered inactive and hence the bacteria do not self-associate. Since *S.* Paratyphi C does not cause large-scale persistent epidemics of typhoid fever, this observation supports the possible importance of type IV pili-mediated bacterial self-association to typhoid fever (Tam *et al.*, 2004).

3. Sensing environmental Mg^{2+}: the PhoP-PhoQ system

The PhoP-PhoQ regulatory system is one of the best-characterised regulons present in enteric bacteria, with the majority of studies having been performed in *Salmonella*. This two-component regulatory system affects the expression of at least 40 proteins (Miller and Mekalanos, 1990). It consists of a sensor anchored in the cytoplasmic membrane (PhoQ) that governs the phosphorylation state of a cytoplasmic gene regulator (PhoP). The periplasmic domain of PhoQ, when bound to Mg^{2+}, promotes the ability of its cytoplasmic domain to dephosphorylate PhoP. Thus, low Mg^{2+} leads to increased levels of phosphorylated PhoP, which subsequently influences the transcription of a host of other genes (Groisman, 2001).

The *phoPQ* operon is regulated in a positive autogenous fashion that amplifies the ability of PhoQ to sense the extracellular concentration of Mg^{2+} (Soncini *et al.*, 1995; Groisman, 2001). Recent work has shown that PhoP activation can occur through self-association of over-expressed PhoP, and this abolishes the need for phosphorylation, and therefore the need for regulation by PhoQ in these conditions (Lejona *et al.*, 2004). In the context of pathogenesis, precise regulation of gene expression by PhoP is crucial. Indeed, *S.* Typhimurium *phoP*-constitutive mutants that exhibit excessive induction and repression of the corresponding PhoP-induced and –repressed genes are as attenuated for virulence in mice as *phoP*-null mutants (Miller and Mekalanos, 1990).

Several PhoP-activated genes (*pag*) as well as PhoP-repressed genes (*prg*) have been identified. Many *pag* genes encode proteins that are involved in remodelling of the outer membrane and in conferring resistance to bile and cationic antimicrobial peptides (Gunn *et al.*, 1998; van Velkinburgh and Gunn, 1999). A number of *prg* genes, on the other hand, are responsible for the formation of the

supramolecular type 3 secretion needle complex for invasion of host cells (Kimbrough and Miller, 2000). Disruption of *pag* or *prg* genes often results in attenuation of virulence (Belden and Miller, 1994). Additionally, the PhoP-PhoQ system regulates the transcription of *mgtC*, a SPI-3 locus that is associated with Mg^{2+} acquisition and is required for virulence (see section C2 of this chapter) (Blanc-Potard and Groisman, 1997). Since a low concentration of Mg^{2+} is indicative of an intracellular environment, it is logical that *Salmonella* should express TTSS genes encoding factors responsible for entry into host cells when Mg^{2+} is not low (Groisman, 2001).

While PhoP-PhoQ is responsible for the regulation of several loci associated with virulence, the majority of PhoP-regulated genes do not play a role in virulence. This is not surprising, given the fact that the PhoP-PhoQ regulatory system is also present in non-pathogenic bacteria (Garcia Vescovi *et al.*, 1994). However, it is interesting that the presence of all PhoP-regulated genes affecting *Salmonella* virulence appear to be the result of horizontal acquisition events. Groisman (2001) hypothesised that evolutionary pressures led to PhoP-PhoQ control of these genes in order to ensure that they would be induced only at the appropriate time and place during the course of infection.

E. Features of host resistance

1. Innate immunity

a. Cells

As described in section B2, PMNs and macrophages are important effectors of the innate immune response. Administration of a monoclonal antibody to Ly6G (a protein found on the surface of PMNs and a small percentage of B cells) rendered mice neutropenic and more susceptible to *Salmonella* infection (Fierer, 2001). Although *Salmonella* preferentially reside in macrophages, these phagocytes play a crucial role in its elimination. Notably, a major difference between innately resistant and susceptible mice is in the ability of their macrophages to kill *Salmonella* (Lissner *et al.*, 1983). This trait has been attributed to a specific locus, *Slc11a1*, which is discussed in section E1d. Although natural killer (NK) cells are considered to be part of the innate immune response, they were shown to modulate the effector function of the adaptive immune response upon challenge in mice previously immunised with attenuated *Salmonella* (Schafer and Eisenstein, 1992). In addition to inducing apoptosis of infected cells, NK cells produce proinflammatory cytokines, including IFN-γ, which activates macrophages and the acquired immune response in the presence of LPS (Kim *et al.*, 2000).

Another type of cell at the interface of innate and adaptive immunity is the DC, which samples antigens at the portals of pathogen entry. Upon exposure to a pathogen (e.g. through stimulation of Toll-like receptors), DCs produce cytokines and chemokines to recruit phagocytes to the site of infection. As DCs mature in response to inflammation, the antigen sampling capacity is down-regulated, the

antigen presenting capacity is up-regulated, and the expression of co-stimulatory molecules is induced (Boss and Jensen, 2003). The DCs then migrate to the lymph nodes to activate the proliferation of antigen-specific T cells. These T cells migrate back to the site of infection, participate in its clearance, and give rise to memory (Mazzoni and Segal, 2004). Indeed, experimental evidence supports a role for DCs in priming naïve T cells (particularly $CD4^+$ T cells) in response to *Salmonella* infection (Yrlid *et al.*, 2001).

b. Toll-like receptors

Central to the innate immune response is the ability to recognise pathogen-associated molecular patterns (PAMPs) such as flagellin, peptidoglycan, LPS, and unmethylated CpG motifs. Recognition of these PAMPs by Toll-like receptors (TLRs) triggers maturation of DCs and the secretion of several cytokines and chemokines (Gordon, 2002). TLRs are integral membrane glycoproteins having an extracellular domain with leucine-rich repeats and a cytoplasmic domain bearing similarity to the signalling domains of IL-1 receptor family proteins (Akira, 2003). Binding of PAMPs to TLRs triggers a signalling cascade that leads to activation of Nuclear Factor-κβ (NF-κβ), which translocates to the nucleus and activates the transcription of a large number of genes involved in immune regulation (Akira and Hemmi, 2003). In addition to inducing the TLR-triggered inflammatory response, NF-κβ is required to induce the expression of co-stimulatory molecules on DCs that allow for complete activation of T lymphocytes (Miyake, 2004).

Bacterial lipoproteins and peptidoglycan are recognised by TLR2 in conjunction with another TLR. Flagellin stimulates the inflammatory response when it binds to TLR5, which is located on the basolateral (but not apical) surface of the

intestinal epithelium (Akira, 2003). Bacterial DNA, which is exposed upon internalisation and degradation, contains unmethylated CpG motifs that are recognised by TLR9, which is found in intracellular compartments (Mazzoni and Segal, 2004). LPS binds to LPS binding protein (LBP) when encountered in the bloodstream. Subsequent to forming a complex with CD14, MD2, and TLR4 on the mammalian cell surface, a potent inflammatory response is initiated. It is worth noting that some mammalian cells discriminate between different LPS structures, and this varies according to the species in question. For example, the anticancer agent taxol binds to TLR4 and mimics the action of LPS in mice, but not in humans. The reverse is true for penta-acylated LPS. Furthermore, human and mouse TLR9 recognise different unmethylated CpG motifs. Such differences are attributed to differences in the structure of the TLRs that bind to these ligands (Akira, 2003).

c. Complement

The complement system is composed of a set of plasma proteins that react with one another to aid in the opsonization and lysis of invading pathogens, as well as to promote the inflammatory response. Many of the components are proteases that become activated upon cleavage. A cascade of reactions is triggered upon interaction of specific complement components with antigen:antibody complexes or surface molecules of pathogens. These initiation events lead to activation of the complement system via the classical pathway, mannan-binding lectin pathway, or alternative pathway. The classical pathway begins with the binding of C1q to antibody complexed with antigen, or direct binding to the pathogen surface. The mannan-binding lectin pathway begins with the binding of mannan-binding lectin to mannose-containing carbohydrates on pathogens. The alternative pathway begins

with spontaneous hydrolysis of C3 and subsequent binding of C3b to the pathogen surface. All three pathways can lead to the formation of the membrane attack complex, which forms holes on the surface of invading pathogens and leads to cell lysis. Additionally, phagocytes possess complement receptors that activate the uptake of pathogens, thus increasing the rate of clearance. Finally, activated complement components, such as C5a, are potent mediators of inflammation that are released into the surrounding area during the chain reaction (Janeway *et al.*, 2001).

Salmonella infection includes a brief stage of bacteremia during which the organism is cleared from the bloodstream before it begins to multiply in the liver and spleen. The O-polysaccharide chain of *Salmonella* prevents insertion of the membrane attack complex; for this reason, the lytic function of complement is thought to be not very important to combating infection by this organism (Mastroeni, 2002). Since activated C3 binds to the terminal sugar of *Salmonella* LPS, opsonisation could play a role in its clearance. Indeed, changes in the terminal sugar structure that affect C3b binding also affect virulence in mice (Fierer and Guiney, 2001). Clearance of *Salmonella* from the bloodstream is greatly delayed in mice whose complement has been depleted by pre-treatment with cobra venom. Furthermore, *C1qa*$^{-/-}$ mice were found to be significantly more susceptible to *Salmonella* infection compared to *C1qa*$^{+/+}$ mice. However, the mechanism of protection in this study remains elusive, since it used a *Salmonella* strain that was resistant to opsonisation and lysis by complement (Warren *et al.*, 2002).

d. *Slc11a1*

The most extensively studied factor of murine innate resistance was originally associated with *S.* Typhimurium, *Leishmania donovani*, and

Mycobacterium bovis BCG infection. This *Ity/Lsh/Bcg* locus was later shown to encode a macrophage protein conferring resistance to all three pathogens and re-named *Nramp1* (for natural resistance associated macrophage protein). After the protein was characterised as a divalent cation transporter localised to the phagosomal membrane, its gene was re-named *Slc11a1* (for solute carrier family 11 member a1) (Wyllie *et al.*, 2002). Although this protein is expressed in PMN as well as macrophages, earlier studies using X-irradiated mice have provided evidence that macrophages rather than PMN are the likely mediators of *Slc11a1*-associated natural resistance (Hormaeche, 1979; Hormaeche *et al.*, 1990; Mastroeni and Sheppard, 2004). This protein is not detected in early endosomal compartments, but in the late endosomal and lysosomal compartments (Gruenheid *et al.*, 1997), suggesting that Nramp1 is not involved in the process of phagocytosis, but that it is directly targeted to the endosomal compartment from the trans-golgi network (Blackwell *et al.*, 2000). Disruption of the *Nramp1* gene has pleiotropic effects that eventually drive the polarity of the T-helper cell response (discussed in section E2b) (Soo *et al.*, 1998).

The mechanism by which Slc11a1 confers resistance has been investigated by focussing on the direction of ion transport into or out of the phagosome, with evidence in support of transport in both directions. These observations gave rise to several hypotheses, among them: 1. Slc11a1 transports iron into the phagosome to serve as a catalyst for the Fenton reaction to generate the extremely reactive hydroxyl radical for bactericidal activity. 2. Slc11a1 salvages iron from the phagosome as part of an iron-recycling pathway. 3. Slc11a1 transports divalent cations out of the phagosome to deprive bacteria of enzyme cofactors necessary for bacterial defence, (Gomes and Appelberg, 1998; Atkinson and Barton, 1999; Kuhn *et al.*, 1999; Mulero *et al.*, 2002).

With so much seemingly contradictory evidence, it would appear that Slc11a1 function is more complex than previously thought. Studies not focussed on the direction of transport have revealed an indirect role for Slc11a1 in the increased expression of inducible nitric oxide synthase (iNOS) and in controlling the maturation of the *Salmonella*-containing vacuole (SCV) (Fritsche *et al.*, 2003; Jabado *et al.*, 2003). *Slc11a1* thus affects macrophage function at the level of bactericidal activity, cytokine production, and antigen presentation (Blackwell, 1996). Interestingly, *Slc11a1* was shown to up-regulate the expression of SPI-2 genes. This highlights the dynamic interaction between the defence and counter-defence mechanisms that have come into play as pathogen and host have co-evolved (Zaharik *et al.*, 2002).

2. Adaptive immunity

a. Antigen processing and presentation

Survival of the mammalian organism requires an immune surveillance that can detect the presence of molecules that have been produced by abnormal host cells or by pathogens. In addition to the recognition of PAMPs by TLRs, the mammalian immune system responds to specific antigens.

All nucleated cells bear major histocompatibility complex (MHC) class I molecules on the cell surface. Phagocytic cells also bear MHC class II molecules. Both types of molecules bind to short peptides and present them as possible antigens for immune surveillance by T lymphocytes. MHC I molecules generally display peptides that originate in the cytosol, such as viral antigens. MHC II molecules generally display peptides of exogenous origin, such as bacterial antigens that have

been phagocytosed (Kaufmann, 1997). Additionally, the CD1 family of molecules can display lipid antigens. Group 1 CD1 molecules display both endogenous and exogenous lipid antigens, while group 2 molecules predominantly display endogenous lipid antigens (Joyce and Van Kaer, 2003).

Cytosolic proteins and antigens are degraded in the proteasome into peptides that are subsequently transported to the lumen of the endoplasmic reticulum (ER) by the heterodimeric transporter associated with antigen processing (TAP). In the ER, peptides are loaded onto MCH I heterodimers within the class I-loading complex, after which MHC I is transported to the cell surface (Joyce and Van Kaer, 2003). Presentation of exogenous antigen by MHC I is known to occur during infections (including *Salmonella* infections) by mechanisms that are not yet clearly defined (Pfeifer *et al.*, 1993; Brode and Macary, 2004).

Group 1 CD1 molecules are assembled in the ER and subsequently targeted to the cell surface through the secretory pathway. Subsequently, they may be recycled between the surface and intracellular compartments multiple times to sample lipid antigens. Group 2 CD1 molecules follow an additional pathway in antigen-presenting cells. Like the MHC II molecule (see below), CD1 associates with Ii in the ER and is transported to the late endosomal/lysosomal compartment. There, Ii dissociates from CD1 in a cathespin-dependent manner and is replaced with lipid antigen, after which CD1 is transported to the surface (Joyce and Van Kaer, 2003).

Exogenous antigens are degraded in the endosome/lysosome. In the ER, newly synthesised MHC II heterodimers associate with Ii chain, which acts as a chaperone to stabilise the molecule, to prevent premature peptide loading, and to direct the molecule to the late endosomal compartment. Ii contains a class II-associated invariant chain peptide (CLIP) that occupies the antigen binding groove after the rest of Ii has been removed by a series of cathespin-catalysed proteolytic

cleavage events. CLIP is then replaced with exogenous peptide by the exchange factor DM, which is targeted independently to the late endosomal compartment. MHC II is then transported to the cell surface (Boss and Jensen, 2003). The *phoP* locus of *Salmonella* (discussed in section D4) influences the efficiency of antigen processing in phagocytes. Macrophages process mutants lacking this locus more efficiently than wild-type *Salmonella.* Furthermore, over-expression of PhoP-activated genes results in poor antigenic presentation (Wick *et al.*, 1995). Since *Salmonella* is known to influence vesicular trafficking, it is not surprising that antigenic processing is also affected (Hernandez *et al.*, 2004).

The MHC genes are the most highly polymorphic genes known; some human MHC I and II genes have as many as 200 alleles. These alleles each occur at a high frequency, such that a given individual is almost certain to be heterozygous. The combination of MHC alleles on a chromosome is referred to as the MHC haplotype. Specific MHC haplotypes have been associated with susceptibility and resistance to *Salmonella* infection in mice (Hormaeche *et al.*, 1985). Similarly, specific MHC haplotypes have been associated with susceptibility and resistance to typhoid fever in humans (Dunstan *et al.*, 2001b).

The amino acid sequence of an MHC II heterodimer in the peptide-binding groove affects its affinity for a given peptide antigen. Thus, MHC heterozygosity is expected to enhance resistance to infectious diseases by increasing the diversity of antigens presented to T lymphocytes. Experiments in mice have indicated that MHC heterozygosity generally confers a selective advantage against infection, although heterozygous offspring are not more resistant to a given pathogen than the resistant parental homozygote (Penn *et al.*, 2002). A recent study assessed heterozygote superiority in the context of murine co-infection with two pathogens associated with opposite MHC haplotype susceptibility profiles. It was revealed that when resistance

associated with a given haplotype is dominant, heterozygotes are more resistant to co-infection than either parental homozygote (McClelland *et al.*, 2003). This mechanism of resistance may help explain the highly diverse nature of MHC loci from an evolutionary standpoint.

b. B lymphocytes and antibodies

B lymphocytes, which originate in the bone marrow, produce antibodies and also act as antigen-presenting cells. These cells internalise antigen that binds to surface Ig, process it, and present it to T lymphocytes in the context of MHC II. Once activated, B cells can proliferate and differentiate into memory and antibody-secreting cells (O'Rourke *et al.*, 1997). Antibody molecules have immense potential diversity that is generated by rearrangements in the genes encoding the light and heavy chains of the antibody molecule (Weigert *et al.*, 1980). Thus, an antibody can be produced to bind nearly any antigen. Cytokines trigger B cells to produce different isotypes of antibody, which differ in their constant region (Davies and Metzger, 1983). IgM is the primary isotype produced in a primary response, whereas and IgG and IgA are predominant later in the response. Furthermore, specific isotypes are associated with T_H1 versus T_H2 responses (Abbas *et al.*, 1996; O'Garra, 1998). Antibodies have multiple roles in host defence: activating complement via the classical pathway, opsonising microoganisms for enhanced uptake by phagocytes, binding and neutralising toxins by blocking their ability to bind receptors, and similarly blocking the ability of pathogens to dock onto host receptors.

The importance of B lymphocytes to protection from *Salmonella* infection has been revealed by experiments with B cell-deficient mice. Igh-$6^{-/-}$ mice (*Slc11a1*$^{s/s}$) were not protected by challenge with virulent *S.* Typhimurium

subsequent to administration of an attenuated (*aroA*) strain that would otherwise generate a protective acquired immune response. Administration of immune serum could not compensate for the deficiency of protection, indicating that a simple lack of antibody production could not generate this phenotype. Furthermore, the T lymphocytes from these B cell-deficient mice exhibited a reduced ability to secrete T_H1 cytokines compared to control mice subsequent to infection with the attenuated *S.* Typhimurium strain. Thus, B cells may play a role in the activation of T cells during the immune response to *Salmonella* (Mastroeni *et al.*, 2000; Ugrinovic *et al.*, 2003).

c. Cell-mediated (T_H1) and antibody-mediated (T_H2) responses

In general, T lymphocytes bearing CD4 molecules recognise MHC II-antigen complexes, while those bearing CD8 molecules recognise MHC I-antigen complexes. Depending on the biochemical signal that is triggered in the T cell, $CD4^+$ and $CD8^+$ T lymphocytes can produce various cytokines. These cytokines can induce or repress the production of other cytokines, as well as induce or repress the activity of other immune cells. This affects the overall immune response. Cytokine-producing $CD4^+$ cells are known as T-helper (T_H) cells. $CD8^+$ cells are primarily responsible for inducing programmed cell death in "altered-self" cells (such as virally infected cells) and these cells are known as cytotoxic T cells (Kaufmann, 1997).

According to which cytokines are produced by the T-helper cells, the outcome can be a T_H1 or T_H2 response. The T_H2 response mainly results in antibody production by B cells, whereas the T_H1 response results in activation of phagocytes and cytotoxic T cells (Kaufmann, 1997). In general, antibodies are effective against extracellular pathogens, while cell-mediated immunity is effective against

intracellular pathogens. Additionally, cytokines that promote a T_H1 response inhibit the T_H2 response, and vice versa (Abbas *et al.*, 1996).

After clearing a *Salmonella* infection, humoral immunity sufficiently protects *Slc11a1*$^{r/r}$ mice, but not *Slc11a1*$^{s/s}$ mice, against subsequent challenge (Eisenstein *et al.*, 1984). In *Slc11a1*$^{s/s}$ mice, cell-mediated immunity (CMI) is thought to be more important than humoral immunity with respect to limiting systemic infections by *S.* Typhimurium. Cytokines characteristic of a T_H1 response are produced in genetically susceptible mice infected with *S.* Typhimurium, and it has been shown that the presence or lack of these cytokines determines how well these mice can combat the infection (Nauciel and Espinasse-Maes, 1992; Mastroeni *et al.*, 1996). This observation is in agreement with the fact that a T_H1 response results in activation of macrophages, which reduces intracellular survival of bacteria, and so, is particularly important for combating such infections (Moulder, 1985; Finlay and Falkow, 1989; Kaufmann, 1993). Furthermore, artificial induction of a T_H2 response has been shown to lead to poorer clearance of *S.* Typhimurium in *Slc11a1*$^{s/s}$ mice (Denich *et al.*, 1993). Interestingly, although *Salmonella* do not enter the cytosol of the cells they inhabit, cytotoxic T cells play a major role in combating *S. typhimurium* infection in mice (Lo *et al.*, 1999).

Although CMI has been shown to be the major effector of immunity to *S.* Typhimurium in *Slc11a1*$^{s/s}$ mice, a study in murine passive immunity showed that both T cells and antibodies are required for resistance to infection (Mastroeni *et al.*, 1993). One study, which found that antibody is required for protection against virulent but not attenuated *S.* Typhimurium, suggested that antibodies delay the rapid accumulation of virulent bacteria before activation of the T_H1 response (McSorley and Jenkins, 2000). It is important to note that the mutation in *Slc11a1* that renders mice genetically susceptible to *Salmonella* infection has not been found in humans

and may be an artefact of mouse inbreeding. Although some alleles of the human *Slc11a1* homologue have been associated with development of an immune response to mycobacteria, no association with typhoid fever has been established (Alcais *et al.*, 2000; Dunstan *et al.*, 2001a).

F. Vaccination

1. Overview

Now more than ever before, humans travel more frequently and to geographically more diverse and remote areas of the world. In addition, individuals visit more places in a shorter time than ever before. These factors favour the spread of infectious diseases (Wilson, 1995). Furthermore, the increase in frequency and spectrum of antibiotic resistance among bacteria places extra emphasis on prophylactic measures for control of infectious diseases (Graham, 2002).

In addition to the composition of the vaccine, the degree and type of protection induced by vaccination varies according to route of administration and dosage. The route by which an individual is immunised affects which phagocytes will encounter and display the antigen, and ultimately affects the nature of the immune response (Thatte *et al.*, 1995). For *Salmonella*, mucosal administration would be most favourable because it is the natural route by which infection occurs (Holmgren *et al.*, 1992). Additionally, low doses of antigen generally induce a T_H1 response, whereas high doses induce a T_H2 response (Abbas *et al.*, 1996).

Some vaccines are used in conjunction with adjuvants, whose function is to enhance and/or bias the immune response. Adjuvants are especially important for

administration of subunit vaccines, since purified antigens are often poorly immunogenic (Greenwood *et al.*, 1991). Currently, the only adjuvants approved for use in humans are aluminium compounds, none of which induce CMI (O'Hagan, 1998). This is partially attributed to the fact that some CMI-inducing adjuvants traditionally used in animals, such as Freund's adjuvant, cause unacceptable side effects (Leenaars *et al.*, 1998). Consequently, candidates for new adjuvants are being tested for CMI efficacy and side effects. These include lipids such as dimethyl dioctadecyl ammonium bromide (DDA) (Lindblad *et al.*, 1997) and monophosphoryl lipid A (Baldridge *et al.*, 2000), as well as the element beryllium, which is known to induce autoimmune T_H1 responses (Lee *et al.*, 2000). Particulate forms, such as liposomes and microparticles, also have promising features as adjuvants. This is not surprising, since the immune system has evolved to respond to particulate matter, such as bacteria and viruses. Microparticles can be administered mucosally and can release antigen over an extended period of time, which abrogates the need for multiple doses. This feature could improve vaccination rates in underdeveloped countries (O'Hagan, 1998).

2. Typhoid fever vaccines

The first vaccines developed against typhoid fever were based on whole *S.* Typhi cells inactivated by heat, acetone, or phenol, but these vaccines caused malaise and fever in many cases (Ivanoff *et al.*, 1994). The moderate protection these vaccines offered lasted for a relatively short period of time (Collins, 1974; Ivanoff *et al.*, 1994).

The first live attenuated *S.* Typhi vaccine was a Ty21a *galE* mutant generated by nitrosoguanidine mutagenesis (Germanier and Furer, 1971). This type of DNA

disruption undoubtedly caused multiple mutations in Ty21a, and it was later found that the attenuating factor was not due to its *galE* mutation, but possibly to its *rpoS* mutation (Hone *et al.*, 1988; Robbe-Saule *et al.*, 1995). Subsequently, it was discovered that the same *rpoS* mutation is present in the parent strain, Ty2 (Robbe-Saule and Norel, 1999). Currently, the Ty21a mutant is the most widely used *S.* Typhi vaccine. It offers protection for up to 7 years in 60% of subjects and causes very little adverse reaction (Ivanoff *et al.*, 1994). However, it requires multiple doses and is ineffective in some cases (Plotkin and Bouveret-Le Cam, 1995).

Another attenuated *S.* Typhi vaccine (CVD908) was subsequently constructed by defined *aroC* and *aroD* deletions in the same parent strain Ty2. These deletions render the strain auxotrophic for aromatic amino acids, which are not present in human tissues at sufficient levels for infection. CVD908 is 100 times more immunogenic than Ty21a, and it can provide good protection with just one dose. However, it caused silent, self-limited vaccinemias when taken in the highest doses (Tacket *et al.*, 1992a; Tacket *et al.*, 1992b). A further deletion in a stress shock protein produced CVD908-*htrA*. This strain generates similar levels of protection and limited side effects, without causing vaccinemia (Tacket *et al.*, 1997b; Tacket *et al.*, 2000). Further trials of CVD908-*htrA* in children in typhoid endemic areas are currently underway. Other promising attenuated Ty2 derivatives are Ty800 and χ4073. Ty800 is disrupted at *phoP/phoQ* (described in section D3), while χ4073 harbours deletions in *cya* (adenylate cyclase), *crp* (global regulator), and *cdt* (involved in systemic dissemination). Both were immunogenic and well tolerated in volunteers after a single dose, but Ty800 was more immunogenic than χ4073 (Hohmann *et al.*, 1996; Tacket *et al.*, 1997a).

Attenuated bacteria are able to express *in vivo* induced antigens and persist in the host long enough to induce CMI. Thus, most research into new typhoid fever

vaccines has concentrated on live attenuated strains. However, live bacteria may be unsuitable for young children, immunocompromised individuals, or patients taking antibiotics. Furthermore, Ty21a is less immunogenic when administered simultaneously with anti-malarial drugs (Plotkin and Bouveret-Le Cam, 1995; Garmory *et al.*, 2002; Parry *et al.*, 2002).

The currently used *S.* Typhi Vi polysaccharide vaccine is effective with only a single dose, can be administered to children as young as 2 years old, and has mild and infrequent side effects (Plotkin and Bouveret-Le Cam, 1995). This vaccine elicits protection that is mediated by high levels of serum Vi antibody (Robbins and Robbins, 1984; Hessel *et al.*, 1999). Since this vaccine relies solely on humoral immunity and is effective for most people, it is apparent that the majority of the population bears more similarity to genetically resistant (*Slc11a1*$^{r/r}$) mice than to the susceptible (*Slc11a1*$^{s/s}$) mice. Indeed, although most research has been performed in susceptible mice, some investigators argue that resistant mice might be more appropriate (Eisenstein and Sultzer, 1983). Furthermore, the fact that polysaccharide is a T lymphocyte independent antigen (Weintraub, 2003) suggests that a typhoid fever vaccine need not elicit a T_H1 response to confer protection. However, chronic carriers of *S.* Typhi display high levels of serum Vi antibody, whereas only a fraction of acute patients show elevated levels of this antibody (Lanata *et al.*, 1983). This underscores a need to understand the correlates of protection by the human immune system against typoid fever infection. Nevertheless, the current satisfactory protection elicited by the Vi polysaccharide subunit vaccine may not endure in the face of *S.* Typhi evolution. The genetic instability of the chromosomal region that produces this antigen (Bueno *et al.*, 2004; Nair *et al.*, 2004) highlights the need for identifying additional protective antigens in the next generation of vaccines.

3. Candidate antigens for subunit vaccines

Although the whole-cell killed *S.* Typhi vaccine causes unacceptable side effects, it provides a level of protection that is higher than either the live attenuated or Vi subunit vaccine (Engels *et al.*, 1998). Thus, a mixture of antigens can provide good protection. The ideal typhoid fever vaccine should contain a variety of antigens and should stimulate CMI as well as humoral immunity for a genetically diverse human population. Choosing specific antigens that elicit protection with minimal side effects, and subsequently combining them with an adjuvant that induces a T_H1 as well as T_H2 response is a strategy that has been tested for another gastrointestinal pathogen, *Helicobacter pylori* (Sanchez *et al.*, 2001).

Since most of the emphasis on new *Salmonella* vaccine development has been on live attenuated strains, relatively few antigens have been investigated for use in subunit vaccines. Known antigens in the mouse model include LPS, flagellin, fimbriae, porins, outer membrane proteins, heat shock proteins, stress-induced catalase, and AhpC (Kagaya *et al.*, 1992; Taylor *et al.*, 1998; Mastroeni *et al.*, 2001). However, only low-level protection could be induced by immunisation with fractions containing porins, flagella, or polysachharide fraction of LPS (Mastroeni *et al.*, 2001). The main challenge, therefore, is the identification of protective antigens.

A recent study has suggested that proteins with increased *in vivo* expression are ideal candidates for multiple subunit vaccination (Rollenhagen *et al.*, 2004). This is based on the idea that T cells respond to antigens in a dose-dependent manner. Several candidate antigens were identified by quantifying *in vivo* GFP expression from single-copy transcriptional fusions to *Salmonella* chromosomal promoters. Subsequent immunisation of mice with two antigens in Freund's complete and incomplete adjuvant in the first and second injection, respectively, resulted in a level

of protection comparable to a killed whole cell vaccine. The two protective antigens were Mig-14 and SseB, a putative transcription factor and a SPI-2 secreted protein, respectively (Rollenhagen *et al.*, 2004). The cytocolic localisation implied by the putative function of Mig-14 suggests that protective antigens need not be surface-exposed components. This is consistent with an earlier study indicating that most antigenic *Salmonella* proteins are located in the cytosolic fraction (Brown and Hormaeche, 1989).

4. Strategies for antigen selection

The first vaccine generated through the use of modern recombinant DNA technology was the hepatitis B vaccine, containing highly purified capsid protein. Since the advent of genomic technology, several new approaches have provided tremendous insight into microbial pathogenesis and have revolutionised vaccine design. These include genome sequencing, signature-tagged mutagenesis (STM), proteomics, DNA microarrays, *in silico* analysis, and *in vivo* expression technology (IVET). These have led to the identification of candidate antigens for subunit vaccines against *Neisseria meningitidis* group B, *Streptococcus pneumoniae*, *Porphyromonas gingivalis*, *Chlamydia pneumoniae*, and *Staphylococcus aureus* (Adu-Bobie *et al.*, 2003).

While conventional vaccinology relied on the identification of the most abundant antigens during the course of infection, new approaches have enabled the identification of all immunogenic antigens. This is significant because the most abundant proteins are often not suitable vaccine candidates (Adu-Bobie *et al.*, 2003). Another method of identifying antigens is through the use of phage display. As discussed in the following sections, phage display can be a powerful tool for this

purpose. Significantly, it allows the selection of any antigen, including those that are only produced during the course of infection. No studies have been published assessing the use of phage display for novel *Salmonella* antigen identification. The many successful applications of phage display for antigen identification justify further investigation in the context of *Salmonella* vaccine development.

G. Phage display

1. Overview

Phage display is a simple and powerful tool for identification and optimisation of ligands that bind to molecules such as antibodies, proteins, carbohydrates, plastic polymer, or nucleic acids (Messmer *et al.*, 2000). Essentially, phage are engineered to express foreign peptides or proteins as integral parts of the capsid. To achieve this, foreign DNA is cloned into the phage gene that encodes the capsid protein. The displayed peptide or protein provides a means for selection, while the DNA that encodes it provides the means for further analysis. The fact that both the gene and its translation product are physically linked in a unit that can propagate itself makes this a powerful tool for identification and characterisation of ligands (Wilson and Finlay, 1998). The useful properties of phage display continually lead to new applications, such as directed evolution of proteins, targeted gene delivery to mammalian cells and in-vivo screening of peptide libraries (Rajotte *et al.*, 1998; Wilson and Finlay, 1998; Poul and Marks, 1999).

2. Biopanning

Biopanning is a form of artificial evolution that is used for identifying protein ligands. It is achieved by successive rounds of affinity selection from a phage population followed by propagation. Like chromatography, a substrate of choice is used as the solid phase, while a suspension containing a phage library constitutes the mobile, liquid phase. After allowing constituents of the liquid phase to bind according to affinity, the solid support is washed to remove residual unbound particles. Finally, elution of the bound particles results in an enriched population of phage that are subsequently propagated to form a new population for the next round of affinity selection.

Many variables affect the successful panning of phage libraries against antibodies. The antibody titre and the copy number of peptide or protein being displayed by individual phage should be properly balanced. In addition, using phage with a lower display copy number would more likely result in binding due to affinity rather than avidity (Wilson and Finlay, 1998). Additionally, it is important to use a method that minimises non-specific or undesirable binding during biopanning. For example, sequences rich in tyrosine and tryptophan have a tendency to bind to plastic. Furthermore, an approach must be used that minimises the tendency for non-recombinant phage to out-compete recombinant clones during enrichment (Wilson and Finlay, 1998).

Affinity selection enriches for ligands that bind with high specificity, but it does not eliminate all non-specific ligands (Christian *et al.*, 1992). For this reason, biopanning is normally followed by other tests that confirm the binding specificity of an isolated phage clone. These are often various forms of enzyme-linked

immunosorbent assay (ELISA) and/or plaque lifts. After these tests, the clone can be sequenced to reveal the identity of the ligand.

There are several methods for biopanning. Depending on the circumstances, one or more rounds of selection may be used. Typically, Fc-biotinylated antibodies are bound to a streptavidin-coated solid support, which is subsequently incubated with phage in solution. Most often, the solid supports used during biopanning are magnetic beads or polystyrene plates (Folgori *et al.*, 1994; Germaschewski and Murray, 1996). One group first mixed the phage with biotinylated antibody, then captured the antibody-bound phage by incubating the mixture in streptavidin-coated petri dishes (Fack *et al.*, 1997). Christian *et al.* (1992) used magnetic beads in the first round of biopanning, and polystyrene plates in the second round. Another study assessed the merits of using magnetic beads versus polystyrene plates (McConnell *et al.*, 1999). It concluded that panning with antibody-coated magnetic beads captured a higher proportion of phage that bound specifically to the antibodies. It also emphasised the importance of capturing the target phage during the first round of panning.

Using polyclonal sera in biopanning adds another level of complexity, since the concentration and repertoire of antibodies are not defined. Additionally, high levels of reactivity with related bacterial proteins of the phage host can complicate detection of phage fusion proteins (Folgori *et al.*, 1994). Furthermore, polyclonal sera often contains antibodies that cross-react with multiple ligands, especially those rich in proline (Tchernychev *et al.*, 1997).

When trying to isolate disease-specific epitopes, it may become necessary to use sera from healthy individuals as a negative control, or as a means of negative selection (Germaschewski and Murray, 1996; Sioud *et al.*, 1996). Folgori *et al.* (1994) found that repeated attempts at negative selection did not significantly deplete

their library of unwanted ligands, so they used the sera from healthy individuals to screen selected phage in plaque lifts and in ELISA tests before sequencing. They also panned with healthy sera in parallel and compared the number of phage recovered.

Panning and enrichment usually results in one clone dominating the final pool of phage. Messmer *et al.* (2000) devised a method called iterative panning and blocking (IPAB) by which the same original library can be panned repeatedly against the same polyclonal antisera to obtain multiple positive clones. Once a clone has been identified, a blocking ligand is constructed, based on the characteristics of that clone. Next, the original library can be panned again in the presence of the blocking ligand so that the clone with the next highest binding affinity can be isolated, and so on. This is important because an antigen that is recognised by sera may not elicit a protective immune response (Wood and Gallagher, 2000, unpublished results).

3. Random peptide libraries and gene fragment libraries

To identify ligands that bind to antibodies, two types of recombinant phage libraries can be used. These are libraries displaying random, synthetically designed peptides, and gene-fragment libraries. There are several benefits of using random sequences of short peptides. Firstly, continuous epitopes are often six amino acids in length (Fack *et al.*, 1997), so libraries consisting of short peptides should be sufficient to identify this type of epitope. Secondly, the diversity of all possible sequences is better represented in a library of shorter peptides, making it a more complete library. This is important, since there is a practical limit to the number of independent clones which can be generated to make a single library (Wilson and Finlay, 1998).

Perhaps the most interesting feature of synthetically designed peptide libraries is in the ability of the peptides to mimic other types of molecules, such as carbohydrates. One study successfully induced anti-carbohydrate antibodies using phage-displayed peptide mimics (Phalipon *et al.*, 1997). However, displayed peptides that mimic an antigen do not always elicit antibodies against the original antigen. For example, another study panned a random peptide library against a protective monoclonal antibody, originally selected from BALB/c mice, that recognised an undefined, discontinuous, conformational epitope of *Bordetella pertussis* toxin (Felici *et al.*, 1993). After biopanning, binding specificity was confirmed by competition experiments with the original antigen. Three different clones of high-copy recombinant phage displaying the epitope-mimicking peptides were chosen for immunisation of BALB/c mice. However, the mice were not immunised with peptides. A specific response against the displayed peptides was observed for one of the phage clones, but none of the recombinant phage elicited a detectable production of antibody against the original antigen.

If the aim of biopanning is to select phage for the purpose of developing a vaccine against an infectious disease, a library containing gene fragments of the causative agent may be more appropriate, since it will have the capacity to display the original epitopes rather than epitope mimics (Fack *et al.*, 1997). If larger fragments are used in a gene fragment library, it is also possible to generate the conformational and discontinuous epitopes recognised originally by the antibodies.

4. Filamentous phage

Most work on phage display has been done using filamentous phage, as it was the archetype. Later, systems using lambda, T4, and T7 bacteriophages were developed (Rosenberg *et al.*, 1997; Wilson and Finlay, 1998). A drawback of using filamentous phage is that the proteins or peptides being displayed must be compatible with the coupled assembly/export process. Furthermore, a requirement for the host to stay alive precludes the production of filamentous phage displaying products that are toxic to the cell. The lambda, T4, and T7 phage have the advantage of lytic propagation, but these phage display systems are not as well-developed as that of filamentous phage (Wilson and Finlay, 1998). A greater wealth of literature and collective experience may improve the likelihood of success in a biopanning experiment, whose outcome is clearly affected by many different parameters.

The filamentous phages are a large group of viruses that infect Gram-negative bacteria. The majority of phage display studies have used the Ff group, which includes the nearly identical M13, fd, and f1 strains. Filamentous phages infect *E. coli* through the F pilus and are secreted continuously, without killing the host. The single-stranded circular genome of approximately 6400 nucleotides is converted by host enzymes to a double-stranded form upon entry into the cell. Phage genes then can be transcribed and translated, and new copies of the genome are produced by rolling-circle replication. The genome encodes 11 proteins, five of which are structural, three of which are required for DNA replication, and three of which are responsible for directing the assembly and export of new particles. The signals for initiation of DNA replication, termination of RNA synthesis, and packaging are located in a non-coding intergenic region (Russel, 1995; Russel *et al.*, 1997).

The major coat protein, pVIII, encloses the particle with about 2700 molecules of about 50 amino acids each (Figure 1.1). Each of the minor coat proteins

is present at about five copies or less. Proteins pVII and pIX are located at the tip that is extruded first from the host cell, while pIII and pVI are located on the end that attaches to the F pilus. The particle is a tubular structure of approximately 10 × 900 nm whose variable length gives it the flexibility to accommodate a variety recombinant polypeptides (Russel, 1995; Russel *et al.*, 1997).

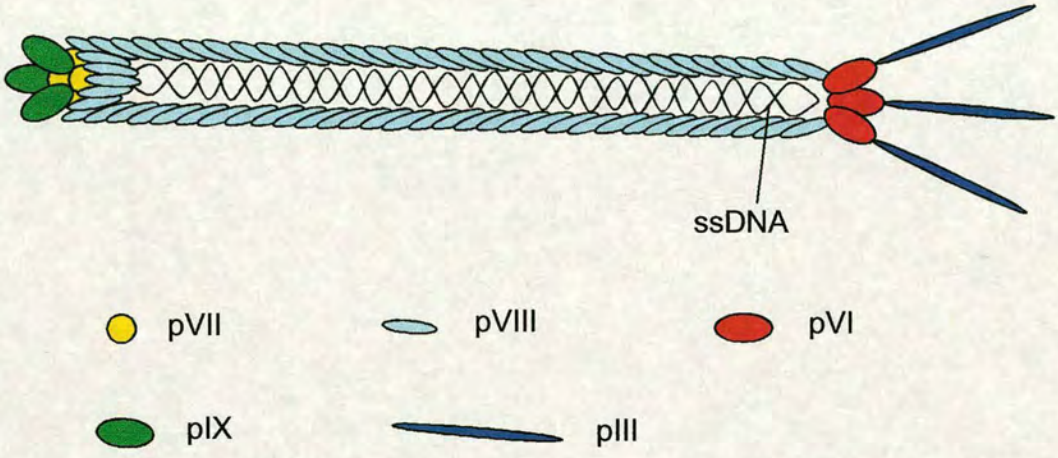


Figure 1.1. Schematic representation of a filamentous phage particle

Although all five structural proteins have been used in phage display, the proteins most commonly used are pIII and pVIII. In both cases, the protein is synthesised with an N-terminal signal peptide that directs it to the periplasm, where it is cleaved before assembly into the new phage particle. Therefore, the introduced polypeptide must be compatible with the assembly and secretion process if it is to be incorporated into the phage particle. The amino terminal residue of both pIII and pVIII are exposed on the phage surface, while their carboxy termini are directed inward. Thus, fusion of foreign DNA to the beginning of the gene results in displayed polypeptide. In addition, the larger size of pIII allows for the display of fusions in the middle region. The smaller pVIII cannot accommodate middle-region fusions. Using pVIII for phage display allows a larger number of recombinant

molecules to be displayed on the surface, increasing the likelihood that binding will occur during biopanning. On the other hand, the low copy number associated with pIII phage display enables discrimination between weak and strong binding affinities in biopanning (Smith and Petrenko, 1997).

Several different systems can be used for pIII and pVIII phage display. Very short peptides, fused to all pIII or pVIII molecules, can be accommodated by the phage particle (Figure 1.2A). Longer peptide- and polypeptide-fusions can be displayed on the phage surface without compromising stability if the respective wild-type protein molecules are also incorporated into the phage particle to achieve a mosaic. This can be accomplished by supplementing a copy of the wild-type gene on the phage genome (Figure 1.2B). Alternatively, mosaic particles can be produced also by using phagemids in combination with helper phage (Figure 1.3) (Smith and Petrenko, 1997).

Phagemids are plasmids that contain the non-coding region of the phage genome as well as the plasmid origin of replication. They can contain a selection marker, such as antibiotic resistance, and a hybrid gene encoding the desired polypeptide-coat protein fusion. Helper phage contain all genes necessary for the infection cycle, typically with mutations that suppress the packaging of helper phage DNA into particles so as to favour packaging of the phagemid instead. When in the host, phagemids can be grown as plasmids. When a helper phage infects this host cell, the phagemid is converted to single-stranded form and is incorporated into a proportion of phage particles. All particles are mosaics on the surface, containing either helper phage ssDNA or phagemid ssDNA (Bass *et al.*, 1990; Smith and Petrenko, 1997).

Typically one or two copies of pIII fusion protein are displayed in the mosaic system, while the number of pVIII fusion proteins on the surface varies greatly. The

proportion of fusion protein molecules incorporated into the particle depends on the size of the insert and the ability of the signal peptidase to process the fusion protein before it can be incorporated into the particle (Malik *et al.*, 1996).

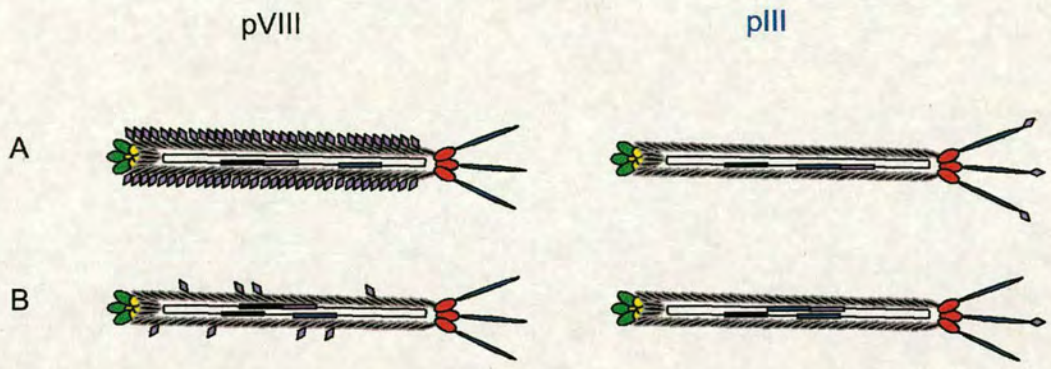


Figure 1.2. Filamentous phage display systems using pVIII and pIII. (A) When foreign DNA is fused to the only copy of the relevant gene, all copies of the resulting protein are identical. (B) When a wild-type copy of the same gene is supplemented on the phage genome, a mosaic particle is produced.

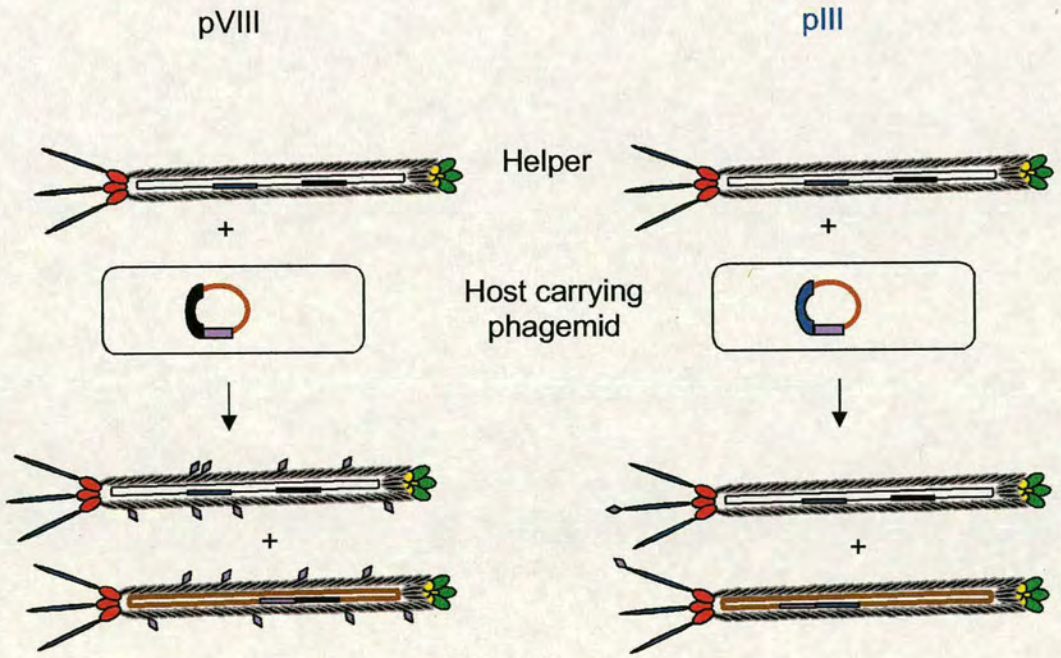


Figure 1.3. Schematic representation of progeny resulting from infection of a phagemid-carrying host by helper phage. All particles are mosaics on the surface, containing either helper phage ssDNA or phagemid ssDNA.

5. Phagemid pG8SAET

A variety of phagemid vectors have been developed and tested by a group studying interactions of *Staphylococcus aureus* proteins with host components (Jacobsson and Frykberg, 2001). Initially, a pIII-based vector was tested by panning a library displaying random fragments of *S. aureus* proteins against IgG and fibronectin. Sequencing the insert DNA from individual clones after selection and enrichment revealed fusions of *S. aureus* genes encoding known IgG- and fibronectin-binding proteins. All were in the correct orientation but in the wrong reading frame. Confrimation that particles bearing these sequences were able to bind specifically to the target led to the conclusion that ribosomal slippage had corrected the reading frame, thus allowing expression of the protein fragment. This led to the hypothesis that phage having correct fusions were somehow less favoured in the selection process, and that ribosomal slippage could be a mechanism of down-regulating the expression of fusion protein. Since multiple rounds of biopanning could not increase the proportion of IgG- and fibronectin-binding clones to more than 40% of the phage population, it was concluded that the level of display may be too low for selection by biopanning. Using a pVIII-based vector, a higher proportion of clones encoding IgG- and fibronectin-binding fragments was obtained. Once again, however, clones with inserts in the wrong reading frame out-competed those with correct fusions (Jacobsson and Frykberg, 2001).

Another group experienced similar problems when biopanning with a library displaying peptides of 40 amino acids in length. Isolation of clones with stop codons and/or frameshifts in the display sequence occurred after panning against some ligands, but not others (Carcamo *et al.*, 1998). Jacobsson and Frykberg thus concluded that decreased expression levels may be advantageous to the display of

some polypeptides. To reduce the reliance of the phage on ribosomal slippage as a means of lowering display levels, the promoter and signal sequence (believed to promote slippage) were replaced, and amber stop codons were introduced downstream of the insert. In the host strain TG1, a proportion of amber stop codons are translated as tryptophan. Thus, 0-3 amber stop codons were added downstream of the insert site in the two vectors containing the old and the new promoter and signal sequence. A stoichiometric mixture of an *S. aureus* library made in each of the 8 vectors was panned against IgG. Three out of four clones isolated subsequently were in pG8SPA1, which was the vector containing one stop codon and the new promoter and signal sequence (Jacobsson and Frykberg, 2001).

The phagemid vector pG8SAET is a derivative of pG8SPA1, with an added sequence encoding an E-tag downstream of the insert. This tag allows screening, using a commercially available antibody, for the expression of fusions in the correct reading frame. Since biopanning with this vector against some ligands resulted in nearly 100% correct clones, the tendency for ribosomal slippage was apparently reduced (Jacobsson and Frykberg, 2001). Thus, the phagemid pG8SAET was selected for use in this study.

AIMS OF THE PROJECT

Phage display has been used successfully in the identification of antigenic components of *Toxoplasma gondii*, *Brugia malayi*, *Plasmodium falciparum*, and a host of other bacterial and viral pathogens (Theisen *et al.*, 2000; Robben *et al.*, 2002; Gnanasekar *et al.*, 2004). Several *Salmonella* antigens have been identified empirically and through screening processes (Brown and Hormaeche, 1989; Huang *et al.*, 2004; Rollenhagen *et al.*, 2004). Only in one recent case has good protection against *Salmonella* infection in the mouse model been demonstrated as a result of the administration of antigenic *Salmonella* subunits (Rollenhagen *et al.*, 2004). This study emphasized the importance of antigen selection based on *in vivo* expressed levels. However, only two protective antigens were identified in this study. A larger number of protective antigens may need to be used for the formulation of a vaccine suitable for a genetically heterogeneous population. No studies employing phage display for the identification of *Salmonella* antigens have been published yet. Phage display is advantageous over other methods of antigen discovery because it is inexpensive and allows the identification of antigens whose expression is restricted to the host environment. The main aim of this project was to identify novel *Salmonella* antigens, using phage display technology, for use in a multiple subunit vaccine. Secondary aims were to identify improved strategies for antigen selection.

CHAPTER 2

Materials and Methods

A. Materials

1. Growth media and solutions

- LB: 1% Difco bacto-tryptone, 0.5% Difco yeast extract, 1% NaCl
- LB agar: LB with 1.5% agar
- LBK: LB agar with 50 μg/ml kanamycin
- LBA50: LB agar with 50 μg/ml ampicillin
- LBA100: LB agar with 100 μg/ml ampicillin
- Top agar: 1% Difco bacto-tryptone, 0.5% Difco yeast extract, 0.5% NaCl, 0.7% agar

- Anion Exchange Buffer 1: 20 mM Tris-Cl (pH 8.0); filter sterilised, de-gassed, and stored at 4°C
- Anion Exchange Buffer 2: same as Anion Exchange Buffer 1, with 1 M NaCl
- Cation Exchange Buffer 1: 50 mM Na_2HPO_4 and 50 mM NaH_2PO_4 combined to pH 7.0; filter-sterilised, de-gassed, and stored at 4°C
- Cation Exchange Buffer 2: same as Cation Exchange Buffer 1, with 1 M NaCl
- Coating Buffer 1: 17.5 mM $NaHCO_3$, 7.5 mM Na_2CO_3, 0.01% NaN_3 (pH 9.5)
- Coating Buffer 2: 50 mM $NaHCO_3$ combined with 50 mM Na_2CO_3 to pH 9.7
- Coomassie Destain: 5% methanol, 7% acetic acid
- Coomassie Stain: 45% methanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue R
- Denaturing Lysis Buffer: 0.1 M NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea (pH 8.0)
- Denaturing Wash Buffer: same as Denaturing Lysis Buffer, with pH 6.3

- Dilution Buffer: 0.25% skimmed milk in PBS-T
- 6× DNA Loading Buffer: 25% sucrose, 0.25% bromophenol blue
- 5 mM dNTPs: 5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP
- Elution Buffer 1: 50 mM sodium citrate, 150 mM NaCl (pH 2.0)
- Elution Buffer 2: 20 mM Tris-Cl (pH 8.0), 0.3 M NaCl, 10% glycerol, 0.1-0.25 M imidazole, 0.5 mM AEBSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin
- 2 × LDS Sample Buffer: 0.5 M Tris-Cl (pH 8.5), 20% glycerol, 1 mM EDTA, 4% LDS, 0.04% Serva Blue G
- Lysis Buffer: 20 mM Tris-Cl (pH 8.0), 0.3 M NaCl, 10% glycerol, 0.1% NP-40, 1 mM imidazole, 0.5 mM AEBSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin
- Lysozyme Solution 1: 10 mg/ml chicken egg white lysozyme in 0.25 M Tris-Cl (pH 8.0)
- MES Buffer: 50 mM MES, 50 mM Tris, 1 mM EDTA, 0.1 % SDS (pH 7.3)
- MOPS Buffer: 50 mM MOPS, 50 mM Tris, 1 mM EDTA, 0.1 % SDS (pH 7.7)
- NBT Developing Solution: freshly prepared by combining 1 ml of 0.1% nitroblue tetrazolium in 10 mM Tris-Cl (pH 8.8) with 40μl 1 M $MgCl_2$, 100μl of 5 mg/ml BCIP in dimethyl formamide, and 9 ml of 0.5 M Tris-Cl (pH 8.8)
- NuPAGE Transfer Buffer: 25 mM bicine, 25 mM Bis-tris (free base), 1 mM EDTA, 20% v/v methanol
- OPD Developing Solution: freshly prepared to a final concentration of 24mM citric acid, 50mM Na_2HPO_4, 0.012% H_2O_2, and 0.4 mg/ml O-Phenylenediamine
- PBS: 0.15 M NaCl, 16.3 mM Na_2HPO_4, 1.9 mM NaH_2PO_4 (pH 7.0)
- PBS-T: PBS with 0.05% Tween 20
- Refolding Buffer: 0.1 M KCl, 20% gylcerol, 20 mM Hepes (pH 7.9)
- Resolving Gel Buffer: 0.75 M Tris-Cl (pH 8.9), 0.4% SDS

- Running Buffer 1: 1% SDS, 25 mM Tris, 0.25 M glycine (pH 7.5)
- Running Buffer 2: 50 mM Tris, 50 mM Tricine, 0.1 % SDS (pH 8.24)
- Running Buffer 3: 50 mM MOPS, 50 mM Tris, 1 mM EDTA, 0.1 % SDS (pH 7.7)
- SDS Sample Buffer: 80 mM Tris-Cl (pH 6.8), 2% SDS, 0.1 M DTT, 10% glycerol, 0.002% Bromophenol Blue
- Stacking Gel Buffer: 0.25 M Tris-Cl (pH 6.7), 0.4% SDS
- STEP solution: 0.5 % SDS, 50 mM Tris-HCl, pH 7.5, 0.4 M EDTA, 1 mg/ml Proteinase K
- 0.5× TBE: 5 mM Tris, 44.5 mM boric acid, 1 mM EDTA (pH 8.3)
- TBS1: 0.15 M NaCl, 10 mM Tris-Cl (pH 7.4)
- TBS1-T: TBS1 with 0.05% Tween 20
- TBS2-T: 0.15 M NaCl, 10 mM Tris-Cl (pH 8.8) with 0.05% Tween 20
- TE: 10 mM Tris-Cl, 1 mM EDTA (pH 8.0)
- TFB1: 30 mM $KC_2H_3O_2$, 0.1 M RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol (adjusted to pH 5.8 with acetic acid)
- TFB2: 10 mM PIPES, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol (adjusted to pH 6.5 with KOH)
- Transfer Buffer: 25 mM Tris, 0.15 M glycine (pH 8.3), 0.0375% SDS, 20% methanol
- Tris Acetate Buffer: 50 mM Tris, 50 mM Tricine, 0.1 % SDS (pH 8.24)
- Tris Buffer 1: 50 mM Tris-Cl (pH 8.0), 50mM EDTA
- Tris Buffer 2: 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 200 μg/ml RNase A
- Tris Buffer 3: 50 mM Tris-Cl (pH 7.5), 1 mM EDTA

- Tris-equilitrated phenol: Phenol having been extracted once with an equal volume of 0.5 M Tris-Cl, pH 8.0, followed by several additional extractions with an equal volume of 0.1 M Tris-Cl, pH 8.0, until the phenol pH>7.8
- Wash Buffer: 20 mM Tris-Cl (pH 8.0), 0.3 M NaCl, 10% glycerol, 20 mM imidazole, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl chloride HCl, 5 μg/ml leupeptin, 5 μg/ml pepstatin

AEBSF and IPTG were purchased from Melford. Unless otherwise stated, all reagents were purchased from Sigma.

2. Protein and DNA molecular weight standards

Protein Standard	Approximate Molecular Weight (Da)
albumin (bovine)	66,000
albumin (egg)	45,000
glyceraldehyde-3-phosphate dehydrogenase	36,000
carbonic anhydrase	29,000
trysinogen	24,000
trypsin inhibitor	20,100
α-lactalbumin	14,200

Table 2.1a Protein molecular weight standards for SDS-PAGE (MW-SDS-70L, Sigma)

Standards were prepared and combined according to manufacturer's instructions

Protein Standard	Approximate Molecular Weight (Da)
triosephosphate isomerase	26,600
myoglobin	17,000
α-lactalbumin	14,200
aprotinin	6,500
insulin chain B, oxidised	3,496
bradykinin	1,060

Table 2.1b Molecular weight marker for SDS-PAGE, ultra low-range (M 3546, Sigma).

The marker consisted of a mixture of the above six proteins

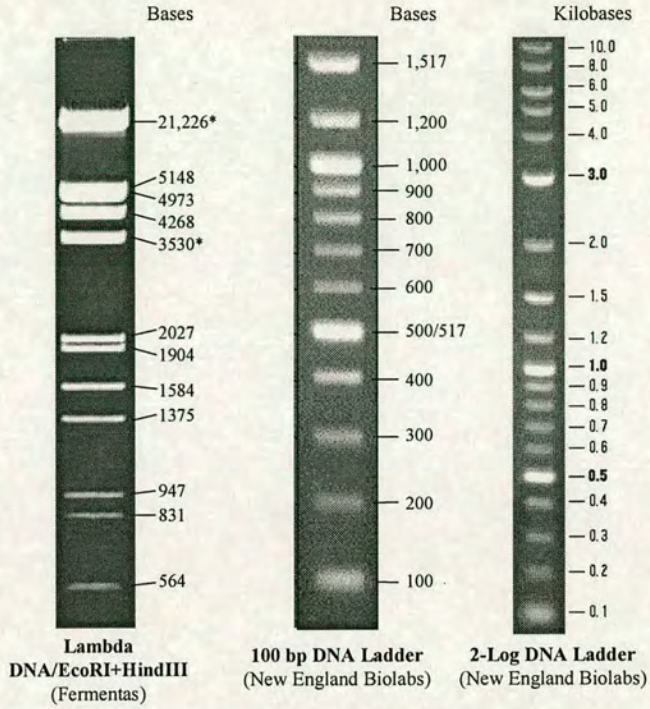


Figure 2.1. DNA molecular weight markers used in this thesis. Cohesive ends of the 12 nt cos site of bacteriophage lambda from fragments 21226 bp and 3530 bp (indicated*) may anneal and form an additional band at 24756 bp. These fragments can be separated by heating at 65°C for 5min and then cooling on ice for 3min.

2. Bacterial strains, plasmids, and primers

Bacterial Strain	Genotype	Source/Reference
E. coli BL21 (DE3)	*hsdS gal* (λ*c*Its857 *ind*1 *S*am7 *nin*5 *lacUV5*-T7 gene1)	Studier *et al.* (1990)
E. coli TG1	*supE hsd* Δ5 *thi* Δ(*lac-proAB*) F'(*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ* ΔM15)	L. Frykberg and K. Jacobsson
E. coli TOP10	F$^-$ *mrcA* Δ(*mrr-hdsRMS-mrcBC*) ϕ80*lacZ* ΔM15 Δ*lac*X 74 *deoR recAI araD139* Δ(*ara-leu*)7697 *galU galK rpsL endAI nupG*	Invitrogen
S. Typhimurium SL1344	*his*	Hoiseth and Stocker (1981)
S. Typhimurium SL3261	*his aroA*	Hoiseth and Stocker (1981)

Table 2.2 Bacterial strains used in this study

Plasmid/*Phage	Relevant Features	Source/ Reference
*M13 R408	defective packaging ability, resulting in preference for plasmid ssDNA over phage ssDNA	Promega
pG8SAET	Amp^R, pUC19 derivative, with *lacZα* removed and the following additions: M13 ori, protein A promoter and signal sequence from *Staphylococcus aureus* in +1 frame, followed by E-tag sequence in +2 frame, followed by 1 TAG, followed by gpVIII of M13 in +2 frame	Jacobsson and Frykberg (2001)
pG8SAET-L7/L12	pG8SAET encoding *S.* Typhimurium *rplL* gene fused, in the correct reading frame, to the signal sequence and E-tag	this thesis
pENTR/SD/D-TOPO	pUC ori, covalently bound topoisomerase I from *Vaccinia* virus at directional cloning site with flanking *att*L1 and *att*L2 sequences, Kan^R	Invitrogen
pET-DEST 42	pBR322 ori; Cml^R and *ccd*B flanked by *att*R1 and *att*R2; T7 promoter, *lac*O, *lac*I, encodes C-terminal V5 epitope followed by 6× His tag, Amp^R	Invitrogen
pET-DEST42 cysA frag	pET-DEST 42 derivative with Cml^R and *ccd*B replaced with fragment of *cys*A	this thesis
pET-DEST42 STM3167 frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *STM*3167	this thesis
pET-DEST42 ytfE frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *ytf*E	this thesis
pET-DEST42 ptsN frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *pts*N	this thesis
pET-DEST42 oat frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *oat*	this thesis
pET-DEST42 yhjJ frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *yhj*J	this thesis
pET-DEST42 gp16 frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *gp*16	this thesis
pET-DEST42 yijO frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *yij*O	this thesis
pET-DEST42 ydiY frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *ydi*Y	this thesis
pET-DEST42 STM2235 frag	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with fragment of *STM*2235	this thesis
pVCN2	pCDNA3-derived, Amp^R, IPTG-inducible T7 promoter encoding eGFP-6×His	V. North, Univ. of Edinburgh
pMOG3	pET-DEST 42 derivative with Cml^R and *ccdB* replaced with *S.* Typhimurium *rplL*	M. Mogensen, Univ. of Edinburgh

Table 2.3 Plasmids used in this study

Primer Name	Primer Sequence	Comments and Usage
cysAfor	5'- CACCATGGATT CTCCTCTACC -3'	PCR of the cysA gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
cysArev	5'- CAGCGGATCG TGATACCAC -3'	PCR of the cysA gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
STM3167for	5'- CACCATGCCG GATGTCAGTC -3'	PCR of the STM3167 gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
STM3167rev	5'- CGACAGATATC CTTCATACTGTTC -3'	PCR of the STM3167 gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
ytfEfor	5'- CACCATGACCA AAGTGGAAC -3'	PCR of the ytfE gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
ytfErev	5'- CATACCCTGTT TGATCATGGG -3'	PCR of the ytfE gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
ptsNfor	5'- CACCATGTTC GACGCCATC -3'	PCR of the ptsN gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
ptsNrev	5'- CAGCGACAG CGTGTGCAG -3'	PCR of the ptsN gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
oatfor	5'- CACCATGCCTG TTGTGGTATC -3'	PCR of the oat gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
oatrev	5'- GCTGTACTTG AGTTTCCCCG -3'	PCR of the oat gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
gp16for	5'- CACCATGTTC CCAAATCAGG -3'	PCR of the gp16 gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
gp16rev	5'- GCGCTTTATAT CTGACGGTTTG -3'	PCR of the gp16 gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
yijOfor	5'- CACCATGTATA TTGATGAACGC -3'	PCR of the yijO gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')

Table 2.4 Primers used in this study. The required 5' CACC for cloning into pENTR/SD/D-TOPO and the introduced start codon have been underlined.

Primer Name	Primer Sequence	Comments and Usage
yijOrev	5'- GATGGCCC CCGTTTTCTG -3'	PCR of the yijO gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
STM2235for	5'- CACCATGCTTG AAAAAAATCAG -3'	PCR of the STM2235 gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
STM2235rev	5'- ACAGCCTGT AGCCGCACG -3'	PCR of the STM2235 gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
ydiYfrac5	5'- CACCATGAAT GCGGGCTAC -3'	PCR of the ydiY gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
ydiYfrac3	5'- GGTACGTTGT CCATACCAGG -3'	PCR of the ydiY gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
yhjJfrac5	5'- CACCATGAAAGT ACGCGACAAAG -3'	PCR of the yhjJ gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (5'-3')
yhjJfrac3	5'- AGTCAAAATG TCAGTATCGG -3'	PCR of the yhjJ gene fragment encoding antigen for cloning into pENTR/SD/D-TOPO (3'-5')
ribfor	5'- GTCTATCACTAAAG ATCAAATCATTGAAG -3'	PCR of rp1L for cloning into pG8SAET (5'-3')
ribrev	5'- AACTTCAACT TCAGCGCCAG -3'	PCR of rp1L for cloning into pG8SAET (3'-5')
sasekv	5'- TATCTGGTGGC GTAACACCTGCT –3'	Colony PCR
etseq	5'- CGTAAACTAGGTGTA GGTATTGCATCTGTAAC -3'	Sequencing of pG8SAET clones (5'-3')
etag	5'- GATCGTCACCCT CGGATCCCTAGG -3'	Colony PCR; Sequencing of pG8SAET clones (3'-5')
oligo1	5'- CGACTCACTATAG GGGAATTGTGA -3'	Sequencing of pET-DEST42 clones (5'-3')

Table 2.4 (continued) Primers used in this study. The required 5' CACC for cloning into pENTR/SD/D-TOPO and the introduced start codon have been underlined.

B. Manipulations of bacteria and phage

1. Manipulations of bacteria

Statonary-phase liquid cultures of bacteria were produced by inoculation of a single colony into 5 ml of LB medium with added antibiotic, if required, and incubated for 16 hours at 37°C with agitation. Larger cultures were produced in the same way, but with an inoculum consisting of a 100-fold dilution of stationary-phase culture. Long-term bacterial stocks were made by combining 1ml of stationary-phase culture with 70 μl of DMSO and immediately storing at -80°C. Bacteria from a liquid culture or freezer stock were propagated on LB agar plates containing antibiotic, if required, by streaking onto the plate and subsequently incubating it at 37°C for 16 hours. Such plates were stored at 4°C for no more than 4 weeks.

2. Manipulations of phage

Helper phage stock was made by the addition of 10^{11} plaque-forming units (PFUs) of M13 R408 helper phage to 10^{8} stationary-phase *E. coli* TG1 host cells in 100 μl of LB. The mixture was incubated at 37°C for 20 min or at RT for 30 min to allow infection, then combined with 5 ml of 42°C top agar, mixed, and poured onto an LB agar plate. After the top agar was allowed to solidify at RT, the plate was incubated at 37°C for 16 hours. The top agar was then removed with a spoon and vigorously mixed with 5 ml of LB, after which the mixture was incubated for 4 hrs at 37°C with agitation. The mixture was then centrifuged at 27,000 × g for 10 min, and

the supernatant was filtered through a membrane with a pore size of 0.45 μm using a syringe. PFUs were estimated by infecting host cells with serial dilutions of the lysate in LB and subsequently plating out with top agar as described above. Alternatively, 10 μl of serial dilutions was spotted onto top agar freshly solidified with 10^8 host cells on an LB plate. Plaques were counted after the plates had been incubated at 37°C for 16 hours, and the number was multiplied by the dilution factor of the lysate. Helper phage stock was stored at 4°C for up to two months, or at -80°C in 0.5 ml aliquots.

The number of M13 particles containing pG8SAET was determined by infecting 50 μl (approximately 10^8 cells) of stationary-phase *E. coli* TG1 with serial dilutions of lysate in 100 μl LB as described above, incubating for 30 min at RT, and spreading over individual LBA50 plates. The plates were incubated at 37°C for 16 hours, after which colonies were counted.

C. Preparation for library construction

1. Purification of DNA from *S.* Typhimurium SL1344

Cells from a 100 ml stationary-phase *S.* Typhimurium SL1344 culture were collected by centrifugation at 2,000 × g for 20 min, and the resulting cell pellet was resuspended in 5 ml of Tris Buffer 1 and frozen at -20°C. Freshly prepared Lysozyme Solution 1 (0.5 ml) was added to the frozen cells, which were subsequently allowed to thaw, with gentle agitation, in a RT water bath. As soon as the cells thawed, they were placed on ice for 45 min, after which 1 ml of freshly

prepared STEP solution was added and mixed. The mixture was then heated to 50°C for 1 hr with occasional, gentle mixing, after which 6 ml of Tris-equilibrated phenol was added and gently mixed for 5 min to form an emulsion. The mixture was then centrifuged at 1,000 × g for 15 min and the upper, aqueous layer was transferred to a clean tube, while the material at the interface of the two layers was carefully excluded. An amount of 3 M sodium acetate (pH 5.4), equivalent to 0.1 of the volume of transferred aqueous layer, was added and gently mixed. Two volumes of ethanol were then added and mixed by inversion. The resulting precipitate, which contained DNA and RNA, was spooled out with a small glass rod. The precipitate was gently rotated against the side of the tube and subsequently allowed to air-dry on the rod. The rod was then transferred to a clean tube containing 5 ml of Tris Buffer 2, and rocked gently overnight at 4°C. After complete dissolution of the pellet, an equal volume of chloroform was added. After inverting several times to mix, the layers were separated by 15 min of centrifugation at 1,000 × g. The aqueous upper layer was transferred to a clean tube, after which the DNA was precipitated as above with sodium acetate and ethanol. Once again, the DNA was spooled out using a glass rod. After the residual ethanol had evaporated, the pellet was allowed to completely dissolve in 2 ml of Tris Buffer 3 by overnight incubation at 4°C with gentle mixing.

2. Quantitation of SL1344 DNA

DNA concentration was determined by agarose gel electrophoresis. Briefly, various dilutions were made in sterile dH_2O and subsequently combined with 6 × DNA Loading Buffer. These samples were then loaded onto a 110 mm × 150 mm

horizontal gel (1% w/v agarose in 0.5 × TBE) containing 0.5 μg/ml ethidium bromide. The samples, along with a control sample containing a known quantity of DNA, were electrophoresed through the gel in 0.5 × TBE at 100 V for 1.5 hr. The gel was then exposed to UV light (λ=313) and imaged using an Epi Chemi III Darkroom (UVP Laboratory Products). The intensity of a band of SL1344 DNA was compared to the intensity of a known quantity of DNA in the control sample.

3. Preparation of electrocompetent host cells

Two 5 ml cultures of stationary-phase *E. coli* TG1 cells were added to 100 ml LB and incubated at 37°C with agitation until the OD (λ=600) reached 0.6, after which the culture was distributed among four centrifuge pots and incubated for 30 min on ice. The cells were then centrifuged 2,500 × g for 15 min at 4°C, and the supernatant was discarded. The cells were then gently resuspended in 1 L of cold 10% glycerol, and centrifuged again. The cycle of gentle resuspension in fresh, cold 10% glycerol and subsequent centrifugation was repeated two more times. After the final centrifugation, cells were gently resuspended in a volume of cold 10% glycerol equal to the pellet, and 50 μl portions were distributed into pre-chilled 1.5 ml tubes. The tubes were snap-frozen in liquid nitrogen before immediate storage at -80°C.

D. Construction of *S.* Typhimurium pG8SAET library

1. Preparation of vector and insert DNA

Stationary-phase *E. coli* TG1 cells containing the vector pG8SAET in 100 ml of LB + 50 μg/ml ampicillin were distributed evenly among 10 sterile tubes, and the plasmid DNA was isolated using Qiagen's QIAprep Spin Miniprep Kit according to manufacturer's instructions. DNA from the 10 plasmid preparations was combined into a single tube. Fifty micrograms of this DNA was digested with 100 units of *Sna*BI (Fermentas) for 2 hrs at 37°C, after which 60 units of calf intestine phosphatase were added, and the mixture was incubated a further 1 hr at 37°C. The digested, dephosphorylated DNA was then extracted 3 times with an equal volume of Tris-equilibrated phenol and twice with chloroform. The aqueous phase containing DNA was then passed through 200 μl of CL 6B sepharose (Amersham Biosciences).

S. Typhimurium SL1344 DNA (100 μg) in 300 μl of dH_2O was sonicated at RT for 10 s, with pulse on 1 s and pulse off 1 s (e.g. 20 seconds total), at 39% amplitude (Sonics & Materials Inc. Vibrason). The DNA was incubated with 0.1 mM dNTPs, 1 mM ATP, 1 × T4 DNA Polymerase Buffer, 25 units of T4 DNA Polymerase and 50 units of T4 Polynucleotide Kinase (Fermentas) at 37°C for 30 min. The mix was extracted 3 times with an equal volume of Tris-equilibrated phenol and 2 times with chloroform, and passaged through 400 μl of CL 6B sepharose (Amersham Biosciences).

2. Ligation and transformation

Five tubes of Ready-To-Go T4 DNA Ligase (Amersham Biosciences), each containing 1-2 μg of cut, dephosphorylated pG8SAET and 3-6 μg of sheared, blunt-ended, phosphorylated insert DNA were incubated for 16 hrs at 16°C. The five ligations were pooled together and combined with 400 μl sterile dH_2O, 4 μl of 10 mg/ml tRNA, and 16 μl of 5 M NaCl. One millilitre of ethanol was added, and the mixture was subsequently inverted several times to mix. After 15 min incubation at RT, the precipitate was pelleted in a microcentrifuge at top speed for 10 min. The supernatant was removed, and the pellet was resuspended once more in 400 μl sterile dH_2O and precipitated again in the same manner. The supernatant was removed, and the pellet was allowed to dry, after which it was resuspended in 11 μl sterile dH_2O. One microlitre was then added to each of 11 tubes containing 50 μl aliquots of electrocompetent host cells having been freshly defrosted on ice. The DNA/ host cell mixture of each tube was then transferred to a pre-chilled cuvette and electroporated at 2.5 V constant, after which the cells of all electroporations were immediately combined in 100 ml of LB. This combined culture was incubated at 37°C for 1 hr with agitation. A small aliquot was then removed for dilution plating, and ampicillin was added to a final concentration of 50 μg/ml. The culture was incubated for 16 hrs at 37°C.

3. Colony PCR and production of phage

Primers "sasekv" and "etag" were used to amplify the flanking regions of the pG8SAET SnaBI restriction site along with any inserted sequence. Ten PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences) were resuspended in 250 µl of dH_2O containing 50 pmol of each primer. Portions of 10 µl were aliquotted into 0.2 ml tubes, and a toothpick was used to transfer cells from a colony to the tube. Cells were resuspended in the mixture well. In a PCR Sprint machine (Hybaid), the tubes were incubated at 94°C for 1 min, followed by 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 5 min. An incubation at 72°C for 5 min followed the last cycle. A 5 µl portion of the reaction was then subjected to electrophoresis on a 1% agarose gel alongside a Lambda DNA/*Eco*RI+*Hind*III Marker (Fermentas) following the method in section C2.

To produce recombinant phage particles, 1.5 ml, 3 ml, and 10 ml portions of the 100 ml culture prepared in section D2 was incubated with 1×10^{11}, 1×10^{11}, and 7×10^{11} helper phage, respectively, for 20 min at 37°C with agitation. This host/phage mixture was then added to 50 ml, 50 ml, and 75 ml, respectively, of molten top agar (45°C), mixed, and poured over 15 plates of LBA50. Once the top agar solidified, the plates were incubated for 16 hrs at 37°C, after which the top agar was scraped off and mixed with an equal volume of LB containing 50 µg/ml ampicillin, vortexed, and incubated for 4 hrs at 37°C with agitation. The mixture was centrifuged at 27,000 × g for 10 min, and the supernatant was filtered through a membrane with a pore size of 0.45 µm. Aliquots of 0.5 ml were stored at -80°C.

E. Production and testing of serum

1. Production of serum

Twenty 8-12 week old female CBA/Ca mice (Division of Bilogy, University of Edinburgh) were injected intraperitoneally (i.p.) each with 2×10^6 stationary-phase *S.* Typhimurium SL3261 in 100 µl of PBS. After 28 days had passed, the same mice were injected i.p. each with 6.5×10^5 stationary-phase *S.* Typhimurium SL1344 in 100 µl of PBS. Twenty-one days after the first challenge with SL1344, the same mice were injected in the same manner with 1.2×10^6 SL1344. Thirty-five days after the second challenge with SL1344, the mice received a final injection of 1×10^6 SL1344. Mice were bled on days 0, 28, 10 post-challenge 1 (pc1), 17 pc1, 10 pc2, and 14 pc3. A final bleed was collected 17 days after the third challenge with SL1344, after which the mice were sacrificed. Blood was allowed to clot in 0.2 ml tubes for 3 hrs at RT, after which the tubes were centrifuged for 10 min at 1,000 × g. The supernatants were combined to form pooled serum samples from each day of collection. Injections were administered by Dr. Maurice Gallagher and serum collection was performed by Dr. Chris Inchley.

2. SDS-polyacrylamide gel electrophoresis, transfer, and immunoblot

a. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially as described by Laemmli (1970). Sample preparation was as follows: 10-12 µl of SDS Sample Buffer containing approximately 6.7×10^7 cells of *S.* Typhimurium SL1344 were boiled for 5 min in 1.5 ml tubes, then placed on ice. Polyacrylamide gel preparation was as follows: a 12.5% resolving gel was prepared by combining 2.5 ml of 30% w/v stock solution having an acrylamide/bis-acrylamide ratio of 37.5:1 (Severn Biotech) with 1.5 ml of Resolving Gel Buffer, 2.5 ml of dH_2O, 50 µl of freshly prepared 10% APS, and finally adding 2.5 µl of TEMED. The mixture was poured into a 5.5 cm × 8.2 cm × 1.0 mm area between two glass plates and overlaid with dH_2O-saturated *sec*-butyl alcohol. After the resolving gel had polymerised, the overlay was poured off. A 5% stacking gel was then prepared by combining 1 ml of the same 30% acrylamide/bisacrylamide solution with 1.5 ml of Stacking Gel Buffer, 3.5 ml of dH_2O, 50 µl of freshly prepared 10% APS, and finally adding 2.5 µl of TEMED. The mixture was poured on top of the resolving gel until it completely filled the remaining area, and a comb was placed into the mixture so that 0.5 cm separated the bottom of the comb from the top of the resolving gel. After the stacking gel polymerised, the comb was removed and the wells were washed with Running Buffer several times until unpolymerised acrylamide had been washed away. The sample and molecular weight marker (MW-SDS-70L, Sigma) were loaded onto a gel, which

was electrophoresed in Running Buffer in a BioRad Mini Protean II cell at 15 mA and 200 V for 1-1.5 hr at RT.

b. Transfer and immunoblot

Three pieces of 6 × 9 cm filter paper, pre-soaked for 5 min in Transfer Buffer, were stacked on top of one another and placed onto the anode of a BioRad TransBlot SD Semi-Dry Transfer Cell. A 6 × 9 cm piece of Protran nitrocellulose membrane with a pore size of 0.45 μm (Schleicher & Schuell), pre-soaked for 5 min in Transfer Buffer, was placed on top of the three soaked filter papers. A gel was then removed from the cell and glass plates, and subsequently carefully placed on top of the nitrocellulose, such that no air bubbles were trapped between the gel and membrane. Three more 6 × 9 cm soaked filter papers were placed on top of the gel, after which an identical stack was prepared adjacent to the first stack using the other gel. The cathode lid was placed firmly on top of the stacks, and proteins were transferred at 500 mA, 15 V for 40 min at 4°C. Membranes were incubated in 0.2% Ponceau S in 1% acetic acid for 5 min, then rinsed with dH_2O several times until protein bands were clearly visible. The bands of the molecular weight marker were traced over with a pen, after which the membrane was cut into three parts. From this point forward, each part of the divided membrane was treated separately. They were further rinsed multiple times with TBS1 until the Ponceau S stain disappeared. Membranes were then incubated overnight at 4°C in 5% skimmed milk in TBS1. Subsequently, they were rinsed twice more with TBS1-T and then incubated for 1 hr at RT in 5 ml of a dilution of mouse serum prepared in 0.5% skimmed milk in TBS1-

T. The membranes were then washed with TBS1-T 3 times for 5 min, after which they were incubated in 5 ml of the same buffer containing 0.5% skimmed milk and a 1:1,000 dilution of alkaline phosphatase-conjugated rabbit anti-mouse antibody for 1 hr at RT. Following this treatment, the membranes were washed 3 times for 5 min in TBS2-T. During this time, the NBT Developing Solution was prepared. Each piece of membrane was then incubated in developing solution for exactly 2 min, after which they were rinsed immediately with tap water to stop the reaction. Membranes were allowed to dry, after which a PowerLook III scanner (UMAX) was used to save the images electronically.

3. Enzyme-linked immunosorbent assay (ELISA)

Cytosolic proteins were isolated from stationary-phase *S.* Typhimurium SL1344 by Z. Carpenter. Briefly, cells from a 500 ml culture were pelleted at 4°C for 15 min at 2,500 × g and subsequently resuspended in 20 ml cold Lysis Buffer, after which they were lysed using a French press. The lysate was then ultracentrifuged at 100,000 × g for 1 hr at 4°C, after which the supernatant was transferred to a fresh tube for a second, identical centrifugation. The supernatant, containing cytosolic proteins, was stored in 0.5 ml aliquots at -20°C. Protein content was quantified using Coomassie Plus Protein Assay Reagent (Pierce) according to manufacturer's instructions.

In each of 96 wells of an Immulon-2 plate, 100 µl of Coating Buffer 1 containing 3.33 µg cytosolic proteins from stationary-phase *S.* Typhimurium SL1344 was incubated overnight at 4°C. Wells were then washed 3 times with 230 µl PBS-T

using an MRW (AM60) plate washer (Dynex Technologies, Inc.). To each well was then added 200 µl Coating Buffer 1 containing 1% skimmed milk, and the plate was kept humid as it was incubated at 37°C for 1 hr. Serial dilutions of serum in Dilution Buffer were then added in 100 µl portions to each well, and the plate was kept humid as it was incubated for 4 hrs at RT. Wells were then washed again 3 times with 230 µl PBS-T. To each well was then added 100 µl of Dilution Buffer containing 1:500 HRP-conjugated anti-mouse IgG antibody (Diagnostics Scotland), and the plate was kept humid as it was incubated at 37°C for 30 min. Wells were then washed again 3 times with 230 µl PBS-T. To each well was then added 100 µl of freshly prepared OPD Developing Solution. The plate was incubated in the dark for 15 min at RT, after which 25 µl of 2 M H_2SO_4 was added to each well to stop the reaction. The absorbance (λ=492) of the wells was then measured using an Anthos 2010 microplate reader and Stingray software.

F. Enrichment of library and screening of selected clones

1. Biopanning conditions

a. Original biopanning conditions

Human plasma (Sigma) (10 µl in 200 µl Coating Buffer 2) was added to a single well of an 8-well Maxisorp Nunc-Immuno Module, which was then wrapped in parafilm and incubated at RT for 1 hr. The well was then washed 8-10 times with

PBS-T over the course of 10 min. To the well was then added either 200 µl of a freshly defrosted aliquot of phage prepared in section D3, or 200 µl of a freshly defrosted aliquot of a *S. aureus* pG8SAET library of phage particles (Jacobsson and Frykberg, 2001). The well was then incubated for 4 hrs at RT with agitation, after which it was washed 30 times over 10 min with PBS-T. Subsequently, 200 µl of Elution Buffer 1 was added to the well and incubated at RT for 3-5 min, after which the elution was transferred to a 1.5 ml tube containing 35 µl of 1 M Tris-Cl, pH 9.0 and mixed. Dilutions of this mixture were made immediately in LB, and a 5-100 µl portion of each dilution was added to a fresh tube containing 100 µl of LB and 50 µl of stationary-phase TG1 host cells. Each tube was incubated for 30 min at RT, after which the contents were spread over individual LBA50 plates. The plates were incubated at 37°C for 16 hours, after which colonies were counted. Colonies were then scraped off in 1 ml of LB, to which 10^{10} R408 particles were added. The mixture was then incubated, combined with top agar, and plated onto LBA50 as in section B2. Phage were subsequently recovered from top agar as in section B2 using LB with 50 µg/ml added ampicillin, after which they were used in a second round of biopanning. The second round was identical to the first, with the exception that phage were incubated in the well for 1 hr and more dilute quantities of elution were used to infect host cells for more convenient quantitation.

b. Modified biopanning conditions

For test biopannings with anti-mouse antibody, wells were coated with 2 µl of goat anti-mouse IgG, Fc-specific (Sigma) in 200 µl Coating Buffer 2 for 16 hrs at

4°C. The wells were washed 3 times with PBS, after which 5 µl of anti-*S.* Typhimurium mouse serum in 200 µl PBS was added. Wells were incubated at RT for 1 hr, after which wells were washed 3 times again with PBS. From this point onward, wells were treated as in section F1a and remaining particles were recovered from wells after elution according to the procedure below. For pre-absorption, 4 or 12 wells were coated with 2 µl of pre-immune CBA/Ca serum in Coating Buffer 2 for 16 hrs at 4°C. Library phage were added to each well consecutively and incubated for 10 min (12 wells) or 15 min (4 wells) prior to incubation in a well having been coated with immune serum. Alternatively, non-immune serum (from CBA/Ca mice not used in the production of hyperimmune serum) was added to library phage prior to biopanning. Serum was incubated at 56°C for 30 min to inactivate complement, or was left untreated, prior to addition to library phage. The phage library (200 µl) was combined with 10 µl or 20 µl of serum and incubated at RT for 15 min. prior to addition of this mixture to wells.

For testing the binding capacity of wells, varying amounts of anti-*S.* Typhimurium serum were added to 200 µl of Coating Buffer 2 and incubated for 16 hrs at 4°C. A single round of biopanning was then performed as in section F1a, using phage generated from pG8SAET having no insert instead of the library phage. For testing different blocking solutions, wells were incubated for 1 hr at 37°C in 1% skimmed milk in PBS-T, 1% gelatin from cold water fish skin (Sigma) in PBS-T, or PBS-T alone. Wells were washed 5 times with PBS-T over 5 min, after which freshly defrosted phage prepared in section D3 were added to wells, which were treated from this point onward as in section F1a. In addition, any phage remaining in the well after treatment with Elution Buffer were harvested by direct addition of host cells to the

well. Upon removal of Elution Buffer, 150 µl of LB and 50 µl of stationary-phase TG1 cells were added to the well, followed by a 30 min incubation at RT. Dilutions were then made in LB, which were immediately plated out on LBA50. Plates were incubated for 16 hrs at 37°C and the resulting colonies were counted.

Immunoglobulin G was partially purified from serum collected in section E1 according to the protocol for purification of rabbit IgG from serum, using saturated ammonium sulfate (SAS), as outlined in Barbas *et al.* (2001). Briefly, SAS was added in 3 µl portions to 0.5 ml of serum stirring on ice to achieve a 45% cut. The precipitate was collected by centrifugation and resuspended in 0.5 ml of PBS. This was subsequently dialysed against 200 ml of PBS 4 times in 20 hrs at 4°C, as was the supernatant fraction. Protein concentrations of the IgG-enriched fraction and of the supernatant were determined using the Coomassie Plus Protein Assay Reagent (Pierce) according to manufacturer's instructions.

After all biopanning modifications had been completed, approximately 0.2 mg of IgG-enriched fraction was used to coat a single well for 16 hrs at 4°C. The well was then washed as in section F1a, and 200 µl of 1% skimmed milk in PBS-T was added. It was then wrapped in parafilm and incubated at 37°C for 30 min, after which it was washed again, eluted, neutralised, incubated with host cells, and plated out as in section F1a. Phage remaining in the well after elution were collected as described in section F1b. Resulting colonies were screened for E-tag expression according to section F2. Stationary-phase liquid cultures were prepared from each chosen clone and combined with equal volumes of other clones to form a mixed culture of stationary phase cells. This culture was subsequently used for propagation

of phage particles in top agar, followed by extraction according to section B2 for a second round of biopanning.

2. E-tag detection

Colonies were patched onto a fresh plate of LBA50 in a grid pattern and incubated at 37°C for 5 hrs. A circular Protran membrane with a diameter of 82 mm and pore size of 0.45μm (Scleicher & Schuell) was then placed onto the gridded colonies, then carefully peeled away with sterile forceps from one point at the edge of the plate. The membrane was then placed on a fresh plate of LBA50 such that the sterile side was in direct contact with the agar, without air bubbles. The plate was covered and placed at 37°C for overnight growth. The membrane was then removed from the plate with forceps and transferred, colony side up, onto three metal caps inside a covered glass petri dish with chloroform in the bottom, as shown (Figure 2.2). The membrane was exposed for 5 min to chloroform vapour, then placed into TBS1-T and incubated at 37°C for 20 min with vigorous shaking. A Pasteur pipette was used to wash off remaining colony debris, and the membrane was washed several times with fresh TBS1-T. The membrane was then incubated with gentle agitation at RT for 1 hr in 5 ml of 5% skimmed milk in TBS1-T, after which 5 μl of mouse anti E-tag antibody (Amersham Biosciences) was added. The membrane was incubated a further 1 hr at RT with gentle agitation, and then washed 3 times for 10 min with PBS-T. It was then incubated in 5 ml 1% skimmed milk in TBS-T containing 2 μl of HRP-conjugated anti-mouse IgG antibody (Diagnostics Scotland) for 1 hr at RT. After the membrane was again washed 3 times for 10 min with TBS1-T, it was washed once more with TBS1. The membrane was developed using the

ECL Plus Western Blotting Detection System (Amersham Biosciences) according to manufacturer's instructions, and imaged in an Epi Chemi III Darkroom (UVP Laboratory Products) or exposed to Super RX Fuji medical X-ray film (Fujifilm).

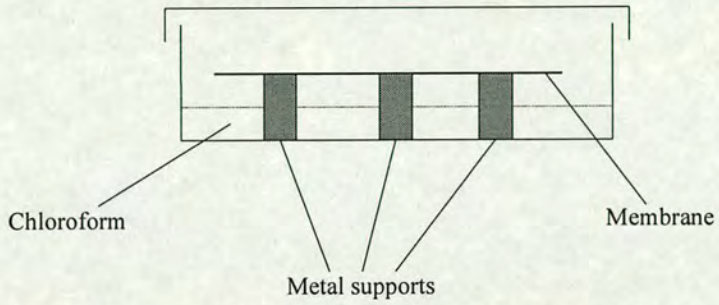


Figure 2.2 Schematic representation of colony lysis for E-tag detection

3. Plasmid purification and sequencing

Small-scale plasmid purification was performed on 5 ml stationary-phase cultures using a Wizard Miniprep Kit (Promega) according to manufacturer's instructions. To sequence the inserts, 20 pmol of primer "etseq" was combined with 0.4 μg of purified plasmid DNA, 2 μl of Sequencing Buffer, and 2 μl of BigDye Terminator v3.1 Cycle Sequencing reaction mix in a final volume of 10 μl. In a PCR Sprint machine (Hybaid), the mixture was incubated at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min and 60°C for 4 min, after which the reaction was stored at -20°C until it was delivered to the Institute of Cell and Population Biology Sequencing Service (University of Edinburgh) for purification using Performa DTR 96-Well Short Plates (Edge BioSystems) and electrophoresis using a 3730 DNA analyzer (Applied Biosystems).

4. Screening of selected clones

a. Positive control clone

Primers "ribfor" and "ribrev" were used to amplify the *rplL* gene from *S.* Typhimurium SL1344 chromosomal DNA prepared in section C1. A 50 µl mixture was prepared, containing 25 ng template DNA, 10 pmol of each primer, 5 µl of the supplied 10 × reaction buffer for *Pfu*, 2 units of *Pfu* DNA polymerase (Promega) and 2 µl of 5 mM dNTPs. The mixture was subjected to thermal cycling according to section D3. A 5 µl portion of the reaction was then subjected to electrophoresis on a 1.2% agarose gel alongside a 100 bp DNA Ladder (New England Biolabs) following the method in section C2. The remaining reaction mixture was then purified using a QIAquick PCR Purification Kit (Qiagen). In a final volume of 50 µl, the purified PCR product was then incubated with 10 units of T4 Polynucleotide Kinase (New England Biolabs) in 1 × reaction buffer supplemented with 1 mM ATP at 37°C for 30 min. The mixture was then incubated at 65°C for 20 min to inactivate the enzyme. A tube of Ready-To-Go T4 DNA Ligase (Amersham Biosciences), containing 1 µg of phosphorylated PCR product and 2 µg of *Sna*BI-digested, dephosphorylated pG8SAET prepared in section D1, was incubated for 3 hrs at RT. The ligation mix (1 µl) was then transformed, according to section D2, into TG1 host cells prepared in section C3. LB (800 µl) was added to the cells and the tube was incubated at 37°C for 1 hr with agitation. Cells were then plated out onto LBA50 and incubated at 37°C for 16 hrs. Resulting colonies were used for PCR according to section D3 and

screened for E-tag expression according to section F2. Finally, a chosen clone was sequenced according to section F3.

b. Dot blots

Stationary-phase cultures were made of each clone to be screened, of which 10 μl was combined with 10^9 PFUs of M13 R408 helper phage in a final volume of 0.5 ml LB+50 μg/ml ampicillin in a 1.5 ml tube. Tubes were incubated for 5 hrs at 37°C with agitation, after which they were centrifuged at maximum speed for 5 min to pellet the host cells. Three hundred microlitres of supernatant were removed and placed into a fresh tube. From this tube, 1 μl was removed and spotted onto Protran nitrocellulose membrane (Schleicher & Schuell). The membrane was then placed in 5% skimmed milk in TBS1-T and incubated for 16 hrs at 4°C with gentle agitation. It was then incubated with agitation at RT for a further 1 hr in either the same solution, the same solution with 10% bovine serum added, or the same solution with 20% foetal bovine serum added. The membrane was then washed 3 times for 10 min with PBS-T, after which it was placed in 5% skimmed milk in TBS1-T containing either a 1:1,000 dilution of mouse anti E-tag antibody (Amersham Biosciences), or a 1:4,000 dilution of mouse anti-SL1344 serum from the final bleed. From this point onward, the membranes were treated identically to the membranes in section F2.

G. Cloning, purification, and assessment of candidate antigens

1. Cloning of selected gene fragments

a. Preparation of heat-shock competent *E. coli* TOP10 cells

Stationary phase *E. coli* TOP10 cells (5 ml) were used to inoculate 500 ml of LB+20 mM $MgSO_4$. Cells were incubated at 37°C with agitation until the OD (λ=600) reached 0.6, after which it was chilled and centrifuged as in section C3. Only two centrifugations were required. The pellet was gently resuspended the first time in 200 ml of cold TFB1 and the second time in 20 ml of cold TFB2. Aliquots of 100 µl in 1.5 ml tubes were snap-frozen and stored as in section C3.

b. Entry vector cloning

PCR was performed using the appropriate pairs of primers from Table 2.3 according to section F4A. Cloning into pENTR/SD/D-TOPO (Invitrogen) was performed essentially according to manufacturer's instructions. Briefly, 1 µl of PCR product was combined with 0.5 µl of Salt Solution (1.2 M NaCl, 60 mM $MgCl_2$), 0.5 µl of TOPO vector, and 0.5 µl of sterile dH_2O. This mixture was incubated at RT for 5 min and subsequently added to a 100 µl aliquot of heat-shock competent *E. coli* TOP10 cells prepared in section G1a, freshly defrosted on ice. The DNA/competent cell mix was incubated on ice for 5 min, after which it was transferred to a 42°C water bath, incubated for exactly 45 seconds, and transferred back to ice for 2 min.

LB (800 µl) was added to the tube, and it was incubated at 37°C for 1 hr with agitation. Cells were then plated out onto LBK and incubated at 37°C for 16 hrs. Resulting colonies were used to inoculate 5 ml cultures for small-scale plasmid purification. Purified plasmid DNA (0.5 µg) was then digested with 5 units of *Bsr*GI (New England Biolabs) in a final volume of 20 µl for 1 hr at 37°C. Half of this reaction was then electrophoresed on a 1.2% agarose gel alongside a 2-Log DNA Ladder (New England Biolabs), essentially according to section C2.

c. Destination vector cloning

Cloning into the pET-DEST42 Gateway Vector (Invitrogen) was performed essentially according to manufacturer's instructions. Briefly, 0.5 µl containing 50 ng of entry vector, 1 µl containing 150 ng of destination vector, 1 µl of 5 × LR Clonase Reaction Buffer, and 1.5 µl of TE were combined in a 1.5 ml tube. After 1 µl of LR Clonase enzyme mix was added, the tube was incubated at 25°C for 1 hr. The reaction was terminated by the addition of 0.5 µl of 10 mg/ml Proteinase K and subsequent incubation for 10 min at 37°C. One microlitre was then transformed, according to section D2, into electrocompetent *E. coli* BL21 (DE3) cells having been prepared according to section C3. After electroporation, 800 µl of LB was added and the tube was incubated at 37°C for 1 hr with agitation. Cells were then plated out onto LBA100 and incubated at 37°C for 16 hrs. Resulting colonies were used to inoculate 5 ml cultures for small-scale plasmid purification according to section F3. Digestion of plasmid DNA was performed according to section G1b. Sequencing was performed using primer "oligo1" according to section F3.

2. Overexpression and detection of His-tagged protein

a. Overexpression

A 0.1 ml portion of stationary phase *E. coli* BL21(DE3) cells harbouring destination vector constructs was used to inoculate 10 ml of LB+100 μg/ml ampicillin. Cells were incubated at 37°C with agitation until they reached an OD (λ=600) of 0.5. IPTG was then added to a 1 mM final concentration, and the cells were incubated with agitation at 37°C for 2 or 4 hrs, 30°C for 3 or 6 hrs, or at 18°C for 18 hrs. Alternatively, a culture was incubated in identical conditions without the addition of IPTG.

b. Detection

The 10 ml cultures in section G2a were centrifuged at 6,000 × g for 10 min. The supernatant was decanted and the cell pellet was resuspended in 0.5 ml of Lysis Buffer. The cells were subsequently placed on ice and sonicated (Sonics & Materials Inc. Vibrason) at 20% amplitude in 3 pulses of 5 s each, with a 1 s gap between pulses. The resulting lysates were centifuged at top speed for 2 min at 4°C in a microfuge. A 0.4 ml portion of the supernatant was transferred to a fresh 1.5 ml tube. The protein content of this supernatant was quantified using the Coomassie Plus Protein Assay Reagent (Pierce) according to manufacturer's instructions. Fifteen micrograms of each sample were combined with dH_2O in a final volume of 5 μl, to which 5 μl of 2 × LDS Sample Buffer were then added, after which the samples were incubated for 10 min at 70°C. Alongside 5 μl of an Ultra Low Range Molecular

Weight Marker (Sigma) in 1 × LDS Sample Buffer, each sample was then electrophoresed in an X Cell II Module (Invitrogen) on a 1.0 mm 12% NuPAGE Novex Bis-Tris Gel (Invitrogen) at 200 V constant for 35 min in MES Buffer. The gel was then fixed by placing it in 100 ml of 40% methanol, 10% acetic acid and incubated for 15 min. Following this, it was washed 2 times for 10 min with dH_2O, and subsequently incubated for 1 hr in 20 ml of GelCode Blue Stain Reagent (Pierce). To destain, it was then incubated in 100 ml dH_2O for 20 min. The gel was incubated at RT with gentle agitation during fixing, washing, staining, and destaining. Finally, it was imaged using an Epi Chemi III Darkroom (UVP Laboratory Products).

3. Purification of expressed His-tagged protein

a. Affinity purification in native conditions

A 500 ml culture of cells were induced according to the conditions in section G2a used to establish the ideal for individual constructs. The entire culture was then centrifuged at 3,000 × g for 10 min at 4°C, and the pellet was frozen at -20°C. It was subsequently resuspended in 20 ml of ice-cold Lysis Buffer. Cells were lysed using a French press at 4,000 psi, and the suspension was centrifuged at 4°C for 30 min at 10,000 × g. In the meantime, 0.1 ml of Ni-NTA agarose beads (Qiagen) was washed twice with 0.5 ml of Lysis Buffer. DNase I was added to the supernatant to a final concentration of 10 μg/ml, after which the mixture was then added to the beads and mixed for 3 hrs at 4°C. The liquid was then allowed to drain away from the beads. The beads were washed 4 times with 1 ml of ice-cold Wash Buffer and sequentially

eluted with 1 ml of ice-cold Elution Buffer 2 containing 0.1 M, 0.15 M, 0.2 M, and 0.25 M imidazole. The protein content in 5 µl portions of each elution was then assessed by electrophoresis and detection according to section D2b.

b. Affinity purification in denaturing conditions

A cell pellet obtained from 500 ml of culture (see section G3a) was resuspended in 20 ml of Denaturing Lysis Buffer and added to 100 µl of Ni-NTA resin. The lysate was allowed to mix with the resin for 3 hrs and was subsequently drained away. The resin was then washed 3 times with 5 ml of Denaturing Wash Buffer, after which it was washed 3 times with 5 ml of Refolding Buffer. Following these washes, bound protein was eluted from the resin with 2 × 0.5 ml of Refolding Buffer containing 0.2 M imidazole. Protein content of the elution was then assessed by electrophoresis and detection according to section D2b.

c. Ion exchange chromatography

The 4 elutions of each protein purified in section D3a were combined, after which the buffer was exchanged for Cation Exchange Buffer 1 or Anion Exchange Buffer 1. Except in the case of the smallest two proteins, this buffer exchange was achieved by dialysis against 3 × 200 ml at 4°C over 20 hrs using Spectra/Por Membrane (Spectrum Laboratories) with a molecular weight cutoff of 3.5 kDa. When necessary, protein solutions were concentrated by sealing the solution within the dialysis membrane and sprinkling polyethylene glycol (PEG, MW = 8,000, Sigma) on top of the membrane to draw out liquid over a period of hours until the

desired volume was achieved. The buffer of the smallest two proteins was exchanged using a Microsep microconcentrator (Filtron Technology) with a molecular weight cutoff of 1 kDa according to manufacturer's instructions. Protein samples were then passed through a Millex-GV 0.22 μm filter (Millipore) to remove debris. Cation or anion exchange chromatography was performed using a Mono S HR 5/5 or Mono Q HR 5/5 column (Amersham), respectively. Samples were loaded in 2 × 5 ml volumes, and the column was washed with 10 ml of the same buffer. Following this, a gradient of increasing salt concentration was passed through the column over 10-30 mls beginning with 100% (Cation or Anion) Exchange Buffer 1 and ending with 100% (Cation or Anion) Exchange Buffer 2. At the end of the gradient, an additional 10 ml of (Cation or Anion) Exchange Buffer 2 was passed through the column. The absorbance (λ=260) of eluate collected in 0.5 ml fractions was used to detect the presence of protein. The protein content in 5 μl portions of selected fractions was then assessed by electrophoresis and detection according to section D2b. Concentration of CysA was achieved by freeze-drying followed by resuspension in a smaller volume.

4. Immunoblot and ELISA of candidate antigens

Purified proteins in 100-200 ng quantities, or 30 μg of cell lysate from section G2b, were subjected to electrophoresis on a NuPAGE gel according to section G2b. At the end of electrophoresis, the gel was not fixed. Instead, it was transferred to a 7 × 8.4 cm Immun-Blot PVDF membrane with a pore size of 0.2 μm (BioRad) using an X Cell II Blot Module (Invitrogen). The membrane was immersed in methanol

and subsequently in Transfer Buffer for 5 min prior to transfer. The same arrangement of filter paper-membrane-gel-filter paper was set up as in section E2b, but NuPAGE Transfer Buffer was used instead of Transfer Buffer. In addition, three blotting pads, soaked in NuPAGE Transfer Buffer, separated the 'sandwich' from the cathode on one side and the anode on the other side, with the membrane closer to the anode than to the cathode. The upper chamber was then filled with NuPAGE Transfer Buffer and the lower chamber with dH_2O. Proteins were transferred at 30 V constant for 30 min at RT, after which the membrane was stained with Ponceau S, marked, and destained as in section G2b. The membrane was then incubated in a 1:4,000 dilution of mouse anti-SL1344 serum from the final bleed in 1% skimmed milk in TBS1-T for 1 hr at RT. Alternatively, the membrane was incubated in a 1:1000 dilution of HRP-conjugated His-probe H-3 mouse monoclonal antibody (Santa Cruz Biotechnology). From this point onward, the membranes were treated identically to the membranes in section F2. Since the His-probe antibody was HRP-conjugated, no secondary antibody was necessary. ELISA was performed according to section E3, except that the wells were coated with 0.1 μg of purified protein.

CHAPTER 3

Construction of a Phage Display Library from *S.* Typhimurium DNA and Production of High-Titre *S.* Typhimurium-Reactive Serum

A. Introduction

The merits of multiple subunit vaccination have been discussed with respect to *Salmonella* infection. In order to formulate a new multiple subunit vaccine, suitable antigenic components need to be identified. Phage display can be a powerful tool for the identification of new antigens when used as part of a selection scheme using immune serum. In this chapter, a phagemid library is constructed from *S.* Typhimurium DNA and evaluated for use in subsequent experimental antigen selection. Additionally, hyperimmune *S.* Typhimurium-reactive serum is raised in two different strains of mice. The serum from both groups is assessed for suitability as a ligand for biopanning the library with the goal of obtaining candidate antigens.

To achieve the goal of identifying *S.* Typhimurium antigens, the phagemid vector pG8SAET was used to construct a library from genomic DNA. This vector was specifically developed for the construction of an M13 phage display library from bacterial genomic DNA (final section of Chapter1 details its development) (Jacobsson and Frykberg, 2001). The vector contains a ColE1 origin for replication of double-stranded plasmid DNA in *E. coli*, an M13 origin for the replication of single-stranded DNA for incorporation into phage particles, and a selectable marker conferring resistance to ampicillin (Figure 3.1). Additionally, it contains the bacteriophage M13 gene encoding the major coat protein, gene VIII, as well as a signal sequence allowing the transport of the coat protein to the periplasm for assembly into phage particles. The promoter and signal sequence are derived from the *spa* gene of *S. aureus* encoding protein A (see final section of chapter 1). The cloning site is located at the 3' end of the signal sequence, at the restriction site for

*Sna*BI. When digested with this enzyme, pG8SAET becomes linear and blunt-ended. The vector also encodes a tag (E-tag) that is 5' to gene VIII and in the same reading frame (Figure 3.2). Furthermore, the vector is designed such that the signal peptide is in a different reading frame from the E-tag and gene VIII sequences. The E-tag will be expressed when the frameshift is corrected by inserted DNA, thereby allowing for screening of individual clones whose sequences are in-frame at both the 3' and 5' ends of the inserted DNA. Finally, an amber stop codon located immediately 3' to the E tag is suppressed in the host strain TG1 to allow low-level expression of fusion.

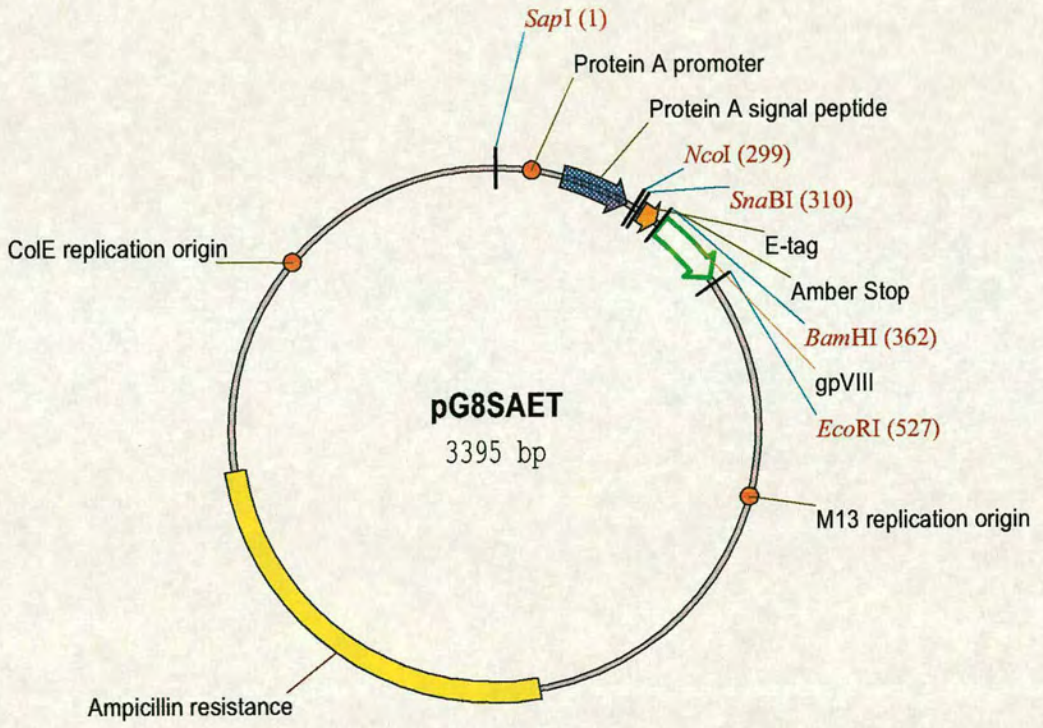


Figure 3.1 General features of pG8SAET, including selected restriction sites.

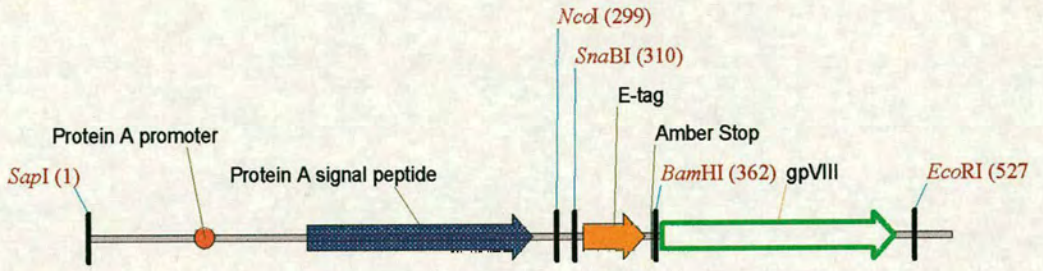


Figure 3.2 Region of pG8SAET designed specifically for phage display library construction.

B. Results

1. Construction of phage display library

a. Preparation of vector and insert DNA

Genomic DNA was purified from *S.* Typhimurium SL1344 by enzymatic lysis and ethanol precipitation. It was then sheared by sonication, made blunt-ended with T4 DNA polymerase, and phosphorylated with T4 polyneucleotide kinase. The vector DNA was digested with *Sna*BI and dephsphorylated with calf intestine phosphatase. After inactivation and removal of enzymes, genomic and vector DNA were subjected to agarose gel electrophoresis (Figure 3.3). The majority (>90%) of genomic DNA fragments ranged between 0.5 and 2.0 kilobase pairs in length.

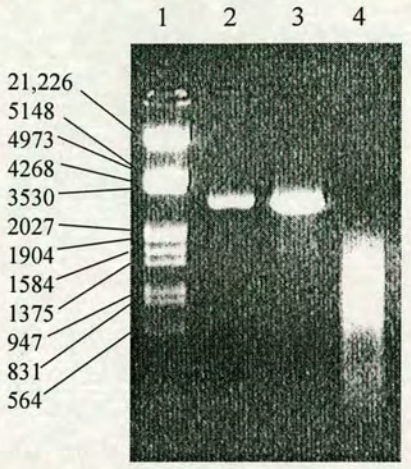


Figure 3.3 Vector and insert DNA used to make the library. The size (in base pairs) of theLambda DNA/EcoRI+HindIII molecular weight maker in lane 1 is indicated to the left. Lane 2: 1 µl of digested pG8SAET. Lane 3: 5 µl of digested pG8SAET. Lane 4: 5 µl of sheared *S.* Typhimurium genomic DNA.

b. Optimisation of ligations for library construction

Five test ligations were performed with the combinations of vector and insert DNA indicated in Table 3.1, after which the ligations were precipitated and resuspended in a total of 10 µl. Five microlitres of each were then subjected to agarose gel electrophoresis (Figure 3.4), and 1 µl of each was transformed into competent TG1 *E. coli* host cells. The number of colonies produced by each transformation, as calculated by dilution plating, are indicated in Table 3.1.

Table 3.1 DNA composition of trial ligations and colonies generated from the transformation of 1/10 of each ligation

Ligation	Vector (0.5 µg/µl)	Insert (0.38 µg/µl)	dH_2O	Colonies from 1/10 of Ligation
1	2 µl	8 µl	10 µl	3.5×10^6
2	2 µl	16 µl	2 µl	4.3×10^6
3	4 µl	8 µl	8 µl	4.2×10^6
4	4 µl	16 µl	---	7.2×10^6
5	4 µl	0 µl	16 µl	2×10^5

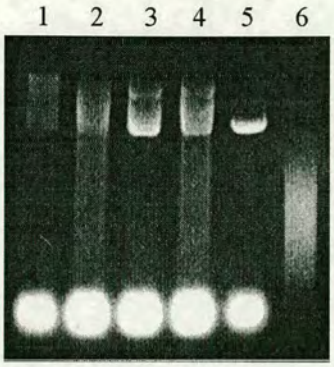


Figure 3.4 Trial ligations for library construction, precipitated with tRNA. Half of each ligation was subjected to agarose gel electrophoresis. Lanes 1-5 correspond to ligations 1-5. Lane 6: 8 µl of insert DNA.

To assess the proportion and size of pG8SAET DNA inserts, colony PCR was performed on 12 colonies produced from the transformation of ligations 3 and 4. The forward primer "sasekv" anneals at approximately 50 bases to the 5' side of the *Sna*BI site, while the reverse primer "etag" anneals approximately 80 bases to the 3' side of it. These reactions were then analysed by agarose gel electrophoresis (Figure 3.5). For colony PCRs, from both sets of ligations, a DNA band was apparent in 11 out of 12 reactions. Additionally, for both sets of PCRs, 9 of the reactions produced a band of larger size than would be produced by a colony with no insert (128 bp). Overall, the average band size of the 9 amplified inserts from ligation 4 was larger than those from ligation 3.

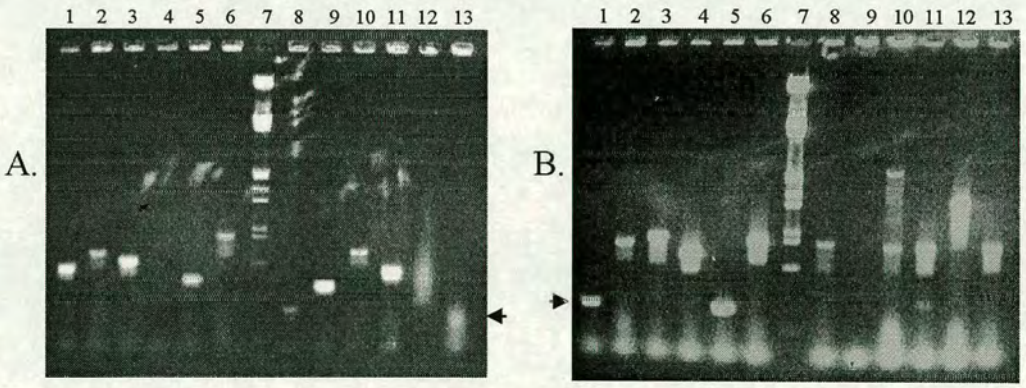


Figure 3.5 A and B. Agarose gel electrophoresis of colony PCRs for transformants from ligations 3 and 4, respectively. Lane 7 is the same molecular weight ladder used in Figure 3.3. Arrowheads represent the position of bands produced by colonies without an insert in pG8SAET.

Finally, colonies from these two ligations were screened for the expression of E-tag by colony lysis on nitrocellulose followed by immunodetection using monoclonal anti E-tag antibody. Two out of 46 colonies from ligation 3 and four out of 80 colonies from ligation 4 were E-tag positive (data not shown).

c. Library construction and analysis

After reviewing the data for ligations 3 and 4, new ligations were prepared as a combination of the two to construct a library for further use. Five ligations were prepared, with two having an identical composition to ligation 3, and the other three having an identical composition to ligation 4. The five ligations were then combined, precipitated twice with ethanol, and the DNA was transformed into electrocompetent *E. coli* TG1 host cells. Dilution plating of the 100 ml culture at one hour after transformation indicated that the culture contained 8×10^8 unique transformants in total. Colony PCR was carried out on 24 of the transformants, and these were analysed by agarose gel electrophoresis (Figure 3.6). Of the 22 reactions that produced a band, 19 were greater in size than the size expected for a colony containing pG8SAET without an insert. E-tag expression was also evaluated for 160 colonies at this stage, of which 7 were positive.

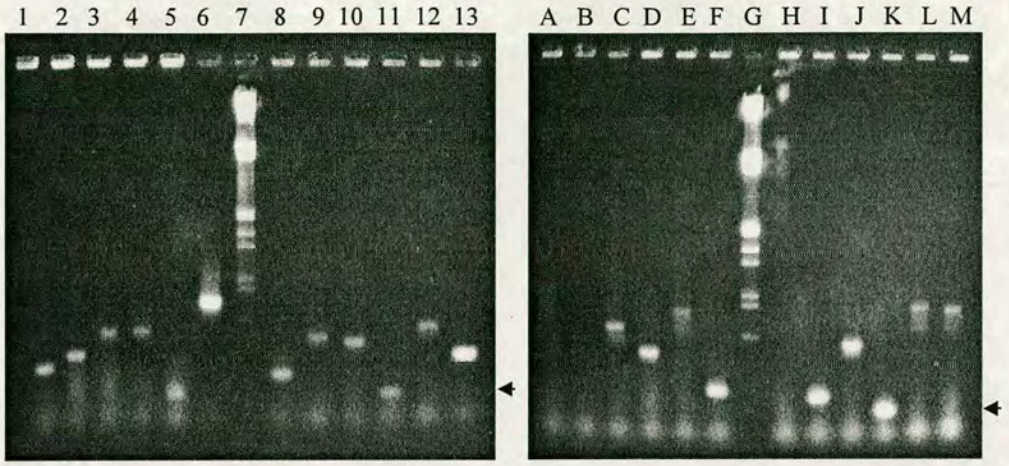


Figure 3.6 Agarose gel electrophoresis of colony PCR performed on randomly selected unique clones from the pG8SAET/*S.* Typhimurium library. Lanes 7 and G contain the same DNA ladder used in Figure 3.3. All other lanes contain colony PCR reactions. Arrowheads represent the position of bands produced by colonies without an insert in pG8SAET.

d. Propagation of pG8SAET library as phage particles

After ampicillin had been added and the 100 ml culture had reached stationary phase, 1.5 ml, 3 ml, and 10 ml portions of the culture were then infected with helper phage at different ratios. The infected library portions were grown in soft agar on antibiotic-containing agar plates, from which phage particles were subsequently extracted and filter-sterilised (see section D3 of Chapter 2 for details). Aliquots of 0.5 ml were stored at -80°C for future use. An aliquot from each of the three infections was then defrosted, serially diluted in LB, and incubated with TG1 host cells. Ampicillin-resistant colonies resulting from the infection represented individual transfers of pG8SAET from a phage particle to a host cell. Phage stocks made from the 1.5 ml, 3 ml, and 10 ml infections were found to contain 1.4×10^{10},

1.7×10^{10}, and 1.0×10^{10} pG8SAET-harbouring particles per ml of frozen stock, respectively. Titration of plaque-forming units (PFUs) from the 10 ml infection indicated that it contained an equal number of particles harbouring helper phage DNA compared to phagemid. E-tag detection performed on 1000-2000 colonies from each of the three infections indicated that 0.2%, 0.3%, and 0.4%, respectively, were positive.

2. Production of high-titre *S.* Typhimurium reactive serum

On day 1, BALB/c (*Slc11a1*$^{s/s}$) and CBA/Ca (*Slc11a1*$^{r/r}$) mice were injected intraperitoneally (i.p.) with SL3261, an attenuated *S.* Typhimurium that is known to confer protection against virulent strains (Hoiseth and Stocker, 1981). The same mice were then injected i.p. with virulent *S.* Typhimurium SL1344 on day 28 and twice again with SL1344 over the course of the subsequent 38 days. Serum from individuals of each mouse strain was collected and pooled together throughout the course of the experiment. Immune sera in different dilutions were used in western blots to probe whole-cell lysates of SL1344, 10 μg cytosolic SL1344 fraction preparation, 0.1 μg purified *S.* Typhimurium GroEL, and 0.6 μg purified *S.* Typhimurium LPS (Sigma) (Figures 3.7-3.9). Immunoblotted membranes were developed with a substrate that stained the membranes directly (see Chapter2 for details). Figure 3.7 and 3.8 compare the BALB/c and CBA/Ca pooled sera, and Figure 3.9 shows the difference in antigen recognition at day28 and day17pc3 of the CBA/Ca antibody response. Figure 3.10 shows the changing CBA/Ca antibody response to cytosolic proteins over the course of the entire experiment, with

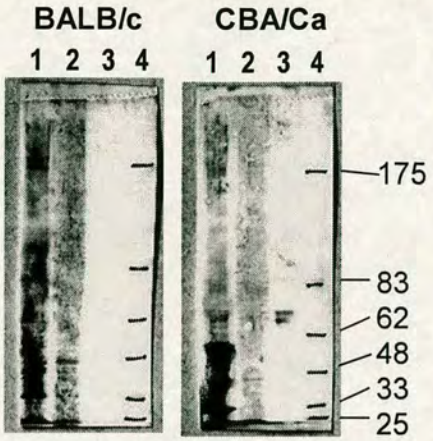


Figure 3.7. Comparison of BALB/c and CBA/Ca sera for LPS and GroEL antibody responses. Final bleeds (day17pc3), at 1:5000 dilution. Lane 1: whole cell lysate; Lane 2: LPS; Lane 3: GroEL; Lane 4: Molecular weight marker, with corresponding sizes (in kDa) on the right.

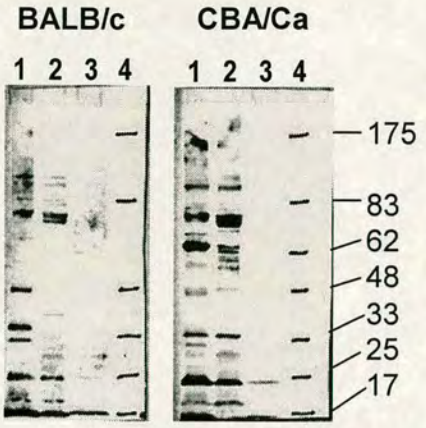


Figure 3.8. Comparison of BALB/c and CBA/Ca sera for strength and diversity of response against proteins. Final bleeds (day17pc3), at 1:10,000 dilution. Lane 1: whole cell lysate; Lane 2: cytosolic proteins preparation; Lane 3: LPS; Lane 4: Molecular weight marker, with corresponding sizes (in kDa) on ther right.

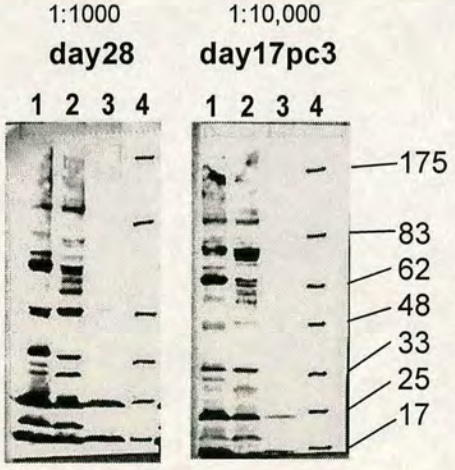


Figure 3.9. Comparison of CBA/Ca sera from different time points during development of the antibody response. Day28 serum was collected 28 days after immunisation with live attenuated SL3261; day17pc3 was the final bleed which was collected 17 days after the third challenge with virulent SL1344. The right panel of this figure is the identical image used in the right panel of Figure 3.8. Lane 1: whole cell lysate; Lane 2: cytosolic proteins preparation; Lane 3: LPS; Lane 4: Molecular weight maker, with Corresponding sizes (in kDa) on the right. Serum dilutions are indicated above each panel.

immunoblotted membranes visualised by ECL. The CBA/Ca response was further investigated by ELISA (Figure 3.10), using the cytosolic preparation as ligand. Endpoints of dilutions were used to determine the strength of anti-*S.* Typhimurium antibody activity in serum samples. The end point of a titration was the lowest

positive reading, where a positive reading is interpreted as an OD (492 nm) value higher than the average of the secondary antibody control wells plus a constant, which was set at 0.2 OD units (Kemeny, 1991).

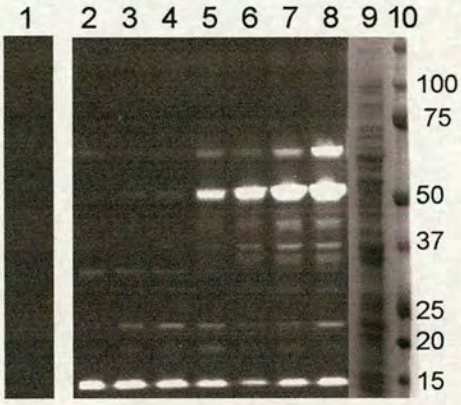


Figure 3.10. Western blots of CBA/Ca sera performed on cytosolic proteins, with corresponding Coomassie-stained gel. A single gel was used for electrophoresis of *S.* Typhimurium SL1344 cytosolic proteins (10 µg/lane), after which it was divided along the pre-stain molecular weight marker (Lane 10; sizes in kDa on the right) for subsequent Coomassie staining (Lanes 9-10) or transfer to nitrocellulose (Lanes 1-8), after which the membrane was cut into strips for Western blot with CBA/Ca sera. Lane 1: day 0; Lane 2: day 28; Lane 3: day 10pc1; Lane 4: day 17pc1; Lane 5: day 10pc2; Lane 6: day 17pc2; Lane 7: day 14pc3; Lane 8: day 17pc3. All sera were used at 1:4000, with the exception of day 0, which was used at 1:200 and exposed with the maximum possible time and setting of the Epi Chemi III Darkroom (UVP Laboratory Products)

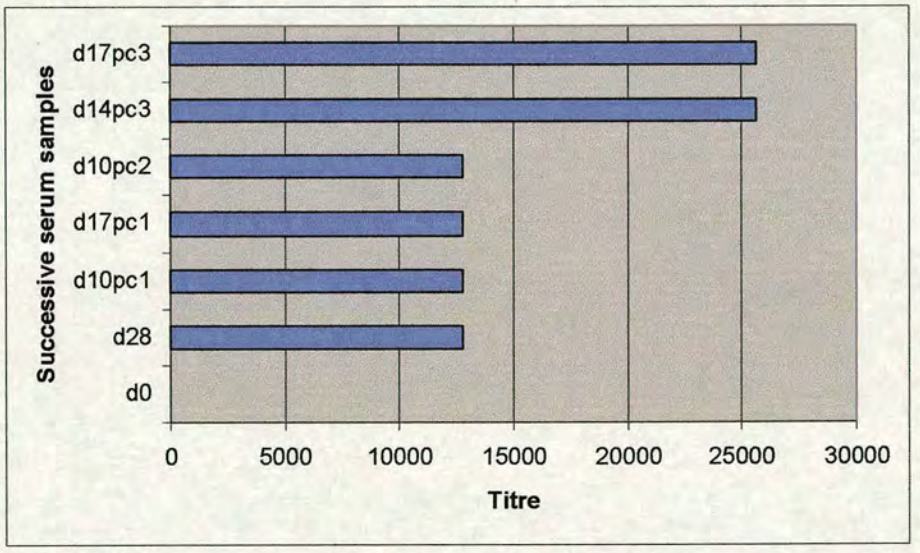


Figure 3.11. ELISA titres from diluted CBA/Ca sera collected on different days after immunisation and challenge with *S.* Typhimurium SL3261 and SL1344, respectively. Each well was coated with 3.33 µg cytosolic fraction of SL1344 and probed with antibody from CBA/Ca mice. d0: pre-immune serum; d28: serum collected 28 days after immunisation with SL3261; d10pc1: serum collected 10 days after challenge 1 with SL1344; d17pc1: day 17 post-challenge 1; d10pc2: day 10 post-challenge 2, etc. Titres represent the serum dilution factor.

Clearly, a strong antibody response was produced in both BALB/c and CBA/Ca mice as indicated by the very dilute level of serum needed for the immunoblot (1:10,000 for the final bleed, day17pc3) and the >25,000-fold increase in the concentration of antibody able to bind to a cytosolic preparation of *S.* Typhimurium proteins (Figure 3.10). Additionally, the difference between BALB/c and CBA/Ca serum is strikingly clear in western blots. These results will be discussed in detail in the following section.

C. Discussion

1. Construction of phage display library

Since pG8SAET was designed specifically as a vector for making filamentous phage libraries from bacterial genomic DNA, it was the most appropriate vector for this study. The library was made using randomly sheared DNA from the virulent *S.* Typhimurium strain SL1344 following the protocol established for pG8SAET (Jacobsson and Frykberg, 2001). Digestion of pG8SAET with *Sna*BI linearised the vector and produced blunt-ended DNA at the digestion site. Subsequent dephosphorylation of the digested vector effectively hindered re-ligation of pG8SAET without an insert (compare ligation 5 to ligations 3 and 4 in Table 3.1). However, complete digestion and/or dephosphorylation of pG8SAET was not achieved, since colonies were indeed obtained from ligation 5. This could be due to random mutation at the restriction site for *Sna*BI or incomplete dephosphorylation by calf intestine phosphatase.

Because shearing is completely random, it is superior to digestion with restriction endonucleases for the purpose of generating genomic DNA fragments for making a library. Shearing SL1344 DNA produced fragments with the majority ranging in length between 0.5 and 2.0 kilobase pairs, which is ideal considering that the average coding sequence in the *S.* Typhimurium genome is approximately 1 kilobase in length (McClelland *et al.*, 2001). Because shearing does not perfectly cut DNA without leaving stretches of single-stranded sequence, mending and phosphorylation of sheared DNA by treatment with T4 polymerase and T4 polynucleotide kinase was necessary. This treatment produced high-quality insert DNA for the purpose of making a library, as evidenced by the large number of transformants produced from ligations 3 and 4 in comparison to ligation 5 (Table 3.1) and the high frequency of pG8SAET clones harbouring an insert as produced from ligations 3 and 4 (Figure 3.5).

Clones produced from ligation 3 have, on average, larger DNA inserts than clones produced from ligation 4 (Figure 3.5). However, the lack of remaining insert DNA in ligation 3 compared to 4 (Figure 3.4) and the higher number of clones produced by ligation 4 (Table 3.1) indicated that ligation 3 had incorporated all the insert DNA without exhausting the availability of digested, dephosphorylated vector. In order to increase the efficiency of ligation and maintain an insert size closer to 1 kb, five ligations were prepared comprising a combination of ligations 3 and 4.

After the library was transformed into electrocompetent *E. coli* TG1 host cells, the total number of individuals contained in the library was determined by serial dilution plating. The library consisted of 8×10^8 individuals with 19 / 22 = 86% (Figure 3.6) containing an insert. Thus, the library contained 6.9×10^8 unique

clones in total. The *S.* Typhimurium genome consists of 4.8×10^6 base pairs. The library contained clones with an average insert size of 500 bp (average size of colony PCR products) – 150 (approximate size of colony PCR product from a colony containing pG8SAET with no insert) = 350 base pairs. The minimum number of clones required for 99% coverage of the genome was calculated using the following formula (Jacobsson *et al.*, 2003): $\ln (1-0.99) / \ln [1-(350 / 4.8 \times 10^6)] = 6.3154 \times 10^4$

However, this formula does not account for the fact that a DNA fragment must be in the correct orientation, as well as the correct reading frame at both the 5' and 3' ends, for proper expression of the gene fragment in pG8SAET. For this reason, the final number required must be multiplied accordingly:

$6.3154 \times 10^4 \times 2$ (orientation) $\times 3$ (correct frame at 5') $\times 3$ (correct frame at 3')

$$= 1.136772 \times 10^6$$

The library of 6.9×10^8 unique clones is significantly larger than theoretically required, which is appropriate given the fact that genomic DNA is not a continuous open reading frame. Nevertheless, the above calculation predicting that 1 in 18 clones will express the E-tag is a good estimation. This is reflected by the fact that the number of clones expressing an E-tag from ligations 3, 4, and the library are 1 in 23, 1 in 20, and 1 in 23, respectively. Interestingly, the proportion of E-tag positive clones dropped sharply after infection of the library with helper phage. This phenomenon has not been extensively investigated, although it was observed as typical for previous libraries constructed using this method (Lars Frykberg, personal communication). It is possible that this is a manifestation of down-regulation of fusion protein expression resulting from infection with the helper phage.

2. Production of high-titre *S.* Typhimurium reactive serum

Most successful screenings of libraries displaying genomic DNA fragments were against a single ligand, a monoclonal antibody, or high-titre serum (Fack *et al.*, 1997; Jacobsson and Frykberg, 2001; Etz *et al.*, 2002). For this reason, it was concluded that serum used for biopanning purposes should be highly enriched for anti-*Salmonella* antibodies. Immunisation of mice with an attenuated strain, followed by multiple challenges with a virulent strain, should progressively enrich the serum of mice for protective antibodies. Additionally, using live bacteria would be better than using lysates because this should mimic natural infection more closely. Furthermore, it would allow for the production of antibodies directed against *in vivo* expressed antigens.

A high level of antibody was raised in the mice as a result of the immunisation and challenges. Although both BALB/c and CBA/Ca mice showed the same overall level of increase in anti-*Salmonella* antibodies as indicated by western blots and ELISA (data not shown), more emphasis was placed on CBA/Ca serum. This is partly due to the fact that BALB/c mice produced a stronger antibody response to LPS than CBA/Ca mice (Figure 3.7). The strong, quenching signal observed in the lanes containing LPS is not unusual for western blots with this molecule, with a ladder-like pattern becoming apparent in some samples. Brown and Hormaeche (1989) investigated immunological profiles of anti-*Salmonella* serum from mice and humans in western blots and ELISAs. The study concluded that while the level of antibodies directed against LPS is substantial, the majority of antigens are likely to be intracellular. The cytosolic fractions tested in Figures 3.8 and 3.9

show a variety of bands. While the study tested a variety of mice with different H-2 haplotypes, CBA/Ca mice were not tested. It is clear in Figure 3.8 that CBA/Ca serum recognises a greater number of protein bands than the BALB/c serum. It is important to note that in this work as well as the study, the bacterial lysate could not contain antigens that are expressed only *in vivo*. Thus, many antigens may not be detected using these methods.

Brown and Hormaeche (1989) also found that some protein bands were more consistently recognised than others. One of the consistently visible bands was deduced to be OmpA, which is now well-documented as antigenic (Singh *et al.*, 2003). Figure 3.8 shows that CBA/Ca serum recognises similar bands that are detected by BALB/c serum, in addition to several additional bands. Since CBA/Ca serum appeared to recognise more antigens and had a slightly lower LPS response than BALB/c serum, this serum was chosen as more suitable for biopanning. All biopannings using hyperimmune serum (discussed in the next chapter) were standardly performed with day17pc3 CBA/Ca serum, which was the final bleed.

Figures 3.9 and 3.10 show that the antigenic profile changed when the CBA/Ca immune system was exposed multiple times to virulent *S.* Typhimurium. Because the growth rates of the attenuated and virulent strains are different in vivo, the regulatory processes governing antigen expression must have been disparate (Clements *et al.*, 2001). Thus, antigens expressed by the attenuated SL3261 strain were likely to have a different profile from that expressed by the virulent strain. However, it is not clear whether the change in profile apparent in Figures 3.9 and 3.10 is due to differential antigen expression and/or to a dynamic host response.

In conclusion, serum with high *S.* Typhimurium reactivity was produced (Figure 3.10), recognising a variety of antigens (Figures 3.8-3.9). Therefore, the CBA/Ca day17pc3 serum to be used in biopanning is expected to be sufficient. The *S.* Typhimurium library constructed in pG8SAET for this study exceeds the theoretical minimum requirements for representing 99% of the genome. Therefore, this library is an appropriate tool for identification of *S.* Typhimurium antigens.

CHAPTER 4

Biopanning and Screening of Phage-Displayed Polypeptides for Antigenicity

A. Introduction

In this chapter, the pG8SAET expression library (constructed in Chapter 3) displaying random *S.* Typhimurium fragments is probed for antigens using the high-titre anti-*S.* Typhimurium serum from CBA/Ca mice (analysed in Chapter 3). The development of the library and serum is described in Chapter 3. As described in Chapter 1, biopanning involves the selection of phage particles from a mixture based on their binding affinity for the ligand, followed by propagation and additional rounds of selection. Jacobsson & Frykberg (2001) used polystyrene micro-wells as the solid support for the ligands used in biopanning with the pG8SAET phagemid vector. A schematic representation of this biopanning method, which was also used in this thesis, is shown in Figure 4.1 below.

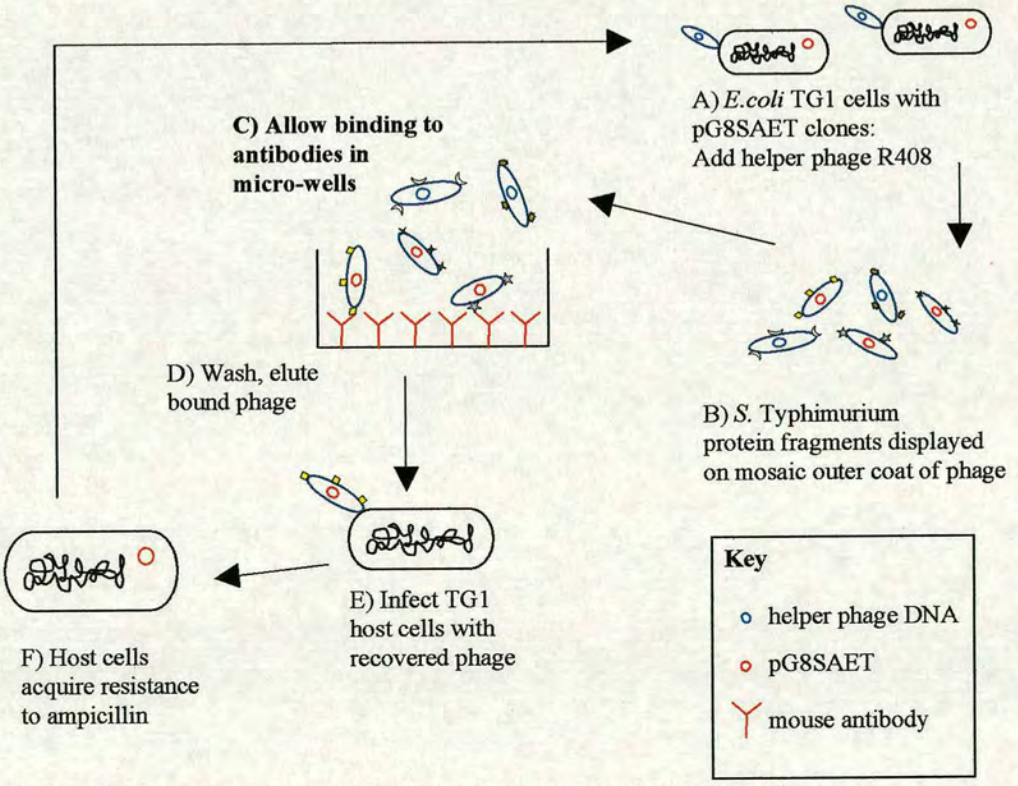


Figure 4.1. Schematic representation of biopanning method used in this thesis

B. Results & Discussion: Biopanning

1. Resuts: Verification of established successful biopanning method

In order to test the biopanning procedure itself, a "control" panning was performed using a pG8SAET library and ligand that have an established successful protocol. An *S. aureus* library made in the vector pG8SAET (kindly provided by Dr. Jacobsson) was panned against human plasma as described previously (Jacobsson and Frykberg, 2001). Essentially, a freshly defrosted aliquot of the phage display library was added to a microwell previously coated with human plasma. The well was washed extensively with PBS-T prior to elution of bound particles using an acidic pH. Eluted particles were allowed to infect *E. coli* host cells, which gave rise to ampicillin-resistant colonies on selective medium. Individual colonies were patched and subsequently transferred onto nitrocellulose, on which they were grown for immunodetection of E-tag expression (see section F2 of Chapter 2 for details). Colonies from the first round of biopanning were collected in a slurry of LB and propagated as phage particles that were used in a second round of biopanning. The *S.* Typhimurium library constructed in pG8SAET was panned against human plasma in parallel as a negative control.

Approximately 6.5×10^4 phagemid were recovered after the first round of biopanning the *S. aureus* library, 35% of which expressed the E-tag. A second round of biopanning particles propagated from the first round yielded 1.2×10^7 phagemid with 89% expressing the E-tag. In comparison, only 1.6×10^3 and 1.7×10^4 phagemid were recovered after the first and second rounds of biopanning the *S.*

Typhimurium library, respectively. E-tag expression for these was at <1% and 9%, respectively. Thus, approximately 40 times more *S. aureus*-derived phagemid bound in the first round compared to *S.* Typhimurium-derived phagemid. The difference between the two was 700 fold in the second round.

2. Discussion: Verification of established biopanning method

In order to establish a starting point for biopanning the *S.* Typhimurium pG8SAET library, it was important to reproduce the successful panning results obtained with another pG8SAET library (Jacobsson and Frykberg, 2001). A significant level of enrichment resulted from biopanning the *S. aureus* library against human plasma, as manifested by the 200-fold increase in total phagemid recovered and near-ubiquitous E tag expression observed after the second round. The *S.* Typhimurium pG8SAET library, whose titre and complexity were comparable to the *S. aureus* pG8SAET library, was only enriched 10-fold in parallel. Additionally, two rounds of panning the *S.* Typhimurium library could not achieve E tag expression levels produced after only one round of panning the *S. aureus* library (9% vs. 35%). This indicates that the first round of biopanning the *S. aureus* library yielded a much more enhanced level of enrichment compared to the other library.

The relative lack of enrichment observed in the *S.* Typhimurium biopanning was expected because numerous serum-binding proteins are produced by *S. aureus* and not by *S.* Typhimurium (Zhang *et al.*, 1999; Zhang *et al.*, 2000a). The ten-fold enrichment observed from the *S.* Typhimurium library was similar to an enrichment of non-specific background binding that occurs in nearly all biopannings (Jacobsson

and Frykberg, personal communication). Hence, although the human plasma used for biopanning may have contained anti-*Salmonella* antibodies, the concentration and/or affinity of such antibodies were not sufficient to produce a significant enrichment from the library constructed in this thesis. In summary, these experiments provide the reference points for positive and negative biopanning results necessary for further experiments in the optimisation of biopanning the *S.* Typhimurium pG8SAET library.

3. Results & Discussion: Optimisation of biopanning conditions

a. Overview

Since the "control" biopanning method that was used with the *S. aureus* pG8SAET library could not provide the same quality of results when applied to the *S.* Typhimurium pG8SAET library panning against serum, some modifications were required. In the following series of experiments, several parameters were assessed in order to achieve a selective enrichment of phagemid clones expressing antigenic *S.* typhimurium epitopes. These parameters included the use of an anti-mouse IgG antibody to capture IgG from the mouse serum, the incubation of library particles in a number of wells coated with pre-immune mouse serum prior to biopanning, the incbation of library particles in solution with untreated or heat-treated non-immune mouse serum, the assessment of coating capacity and blocking agents, serum fractionation for enrichment of IgG, and combinations of these factors. A generalised scheme of the series of biopanning experiments is displayed in Figure 4.2.

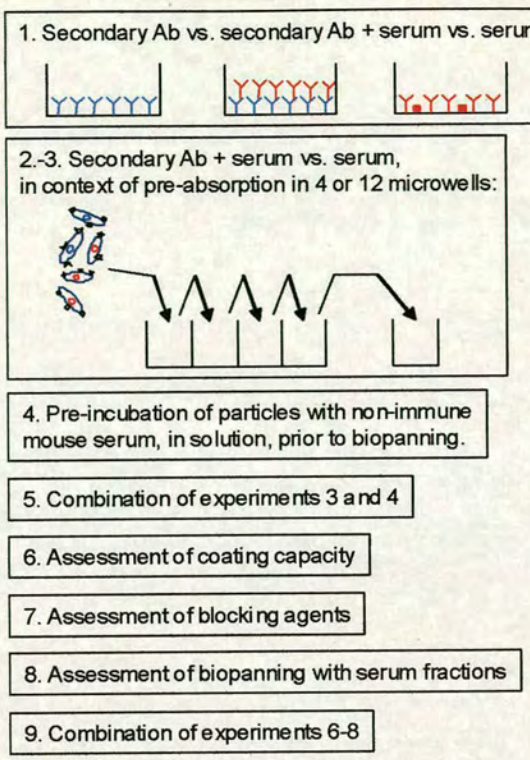


Figure 4.2. Schematic representation of Experiments 1-9

b. Experiment 1: Results

In a parallel setup of biopannings, micro-wells were coated with either goat anti-mouse IgG (Fc) antibody, 5 µl of anti-*S.* Typhimurium serum, or goat anti-mouse Fc followed by 5 µl anti-*S.* Typhimurium serum. After elution, host cells were added to the micro-wells to recover any remaining phage that did not elute. Other biopanning parameters were according to the previously established protocol (Jacobsson and Frykberg, 2001). Phagemid from eluted particles were combined with phagemid subsequently recovered from the corresponding well. These were propagated and used in a second round of biopanning against the same ligand from the first round. The resulting phagemid recoveries and percentage expressing the E-tag are indicated in Table 4.1.

Ligand	Eluted phagemid, in CFUs (% E-tag +)	CFUs of phagemid Recovered from Well (% E-tag +)
ROUND 1		
Anti-mouse Ab	3×10^3 (1%)	600 (2%)
Anti-mouse Ab + serum	5×10^3 (1%)	200 (14%)
Serum	2×10^3 (1%)	300 (8%)
ROUND 2		
Anti-mouse Ab	5×10^6 (20%)	1×10^4 (6%)
Anti-mouse Ab + serum	1×10^5 (28%)	4×10^3 (23%)
Serum	4×10^5 (32%)	7×10^3 (23%)

Table 4.1. Recovery of phagemid from two rounds of biopanning in Experiment 1. CFUs correspond to stage F) of biopanning, indicated in Figure 4.1. The pG8SAET library library was panned against the ligands indicated. Host cells were added to wells after elution in order to recover remaining bound phagemid. The two categories of phagemid obtained from the first round were combined for each ligand and propagated for subsequent selection in the second round against the corresponding ligand. Phagemid CFUs obtained after each round of selection, and the proportion expressing E-tag are indicated.

c. Experiment 1: Discussion

In Experiment 1, biopanning with serum directly coated in microwells was compared to biopanning with the assistance of anti-mouse antibody. In theory, wells pre-coated with antibodies directed against the mouse IgG molecule should promote the selective retention of these antibodies subsequent to addition of the serum. Furthermore, the Fc-specificity of the antibody should aid in orienting the mouse antibodies to maximise exposure of their antigen-binding regions. However, no significant difference was observed when biopanning was performed in wells containing anti-mouse antibody plus serum compared to wells containing either component alone. The similar level of enrichment achieved when panning against serum alone versus anti-mouse antibody alone indicated that the enrichment was unlikely to be specific for *Salmonella* antigens. This is an important point because the enrichment level indicated by the number of recovered phagemid is greater than

200 fold between the first and second round of biopanning. However, the number of phagemid recovered from the first round, as well as the frequency of E tag expression among these, are much lower than what was obtained in the first round of biopanning with the *S. aureus* library. Finally, sequencing of 30 different E-tag positive clones from the second round of biopanning for each of the three categories of ligand failed to reveal any clones encoding known antigens or having overlapping insert sequences (data not shown). Indeed, undesirable enrichment of phage binding to the secondary antibody was documented in another study that used a random peptide library (Messmer and Thaler, 2001). Iterative panning of the peptide library led to the development of a specific peptide for blocking unoccupied sites on the monoclonal secondary antibody, which solved the problem of undesirable binding of phage particles. However, affinity-purified or otherwise polyclonal antibody would require the development of a more complex blocking peptide mixture, specifically designed for the antibody in question.

d. Experiments 2-3: Results

Four (Experiment 2A) or 12 (Experiment 3A) wells were coated with pre-immune CBA mouse serum and an additional well was coated with the day17pc3 CBA/Ca anti-*S.* Typhimurium serum. Another series of wells was set up in parallel, but the wells were coated with anti-mouse antibody prior to addition of mouse serum (Experiments 2B and 3B). The library was sequentially incubated in wells containing pre-immune CBA/Ca serum before addition to the well containing anti-*S.* Typhimurium serum. Phagemid from eluted particles were combined with phagemid

subsequently recovered from the corresponding well. In the second round, phage were not pre-incubated in wells containing pre-immune serum. Instead, phage derived from Experiment A were panned against ligand A in two wells: one with pre-immune and one with anti-*S.* Typhimurium serum, and likewise for B. Only eluted phagemid were counted from the second round. The resulting phagemid recoveries are indicated in Tables 4.2 and 4.3.

Round 1 CFUs		
Ligand	Eluted	Recovered from Well
A. Serum	1.5×10^3	400
B. Anti-mouse Ab + serum	2.5×10^3	500
Round 2 CFUs (from eluted phagemid-containing particles)		
	Pre-immune	Immune
A. Serum	1.2×10^4	1.2×10^4
B. Anti-mouse Ab + serum	9×10^3	7×10^3

Table 4.2. Recovery of phagemid from Experiment 2. CFUs correspond to stage F) of biopanning, indicated in Figure 4.1. Phagemid CFUs obtained after elution and retrieval of remaining bound particles in wells are indicated. The pG8SAET library was pre-adsorbed in FOUR consecutive wells coated with pre-immune serum, or pre-immune serum bound to anti-mouse antibody, prior to biopannning against hyperimmune anti-*S.* Typhimurium serum in round 1. The two categories of phagemid obtained from the first round were combined for each ligand A. and B. and propagated for subsequent selection against the corresponding ligand in the second round. Phage were not pre-adsorbed in the second round but were panned against pre-immune or hyperimmune anti-*S.* Typhimurium serum in the second round. Phage originating from panning against ligand A and B were panned against the respective ligands A and B in the second round.

Round 1 CFUs		
Ligand	Eluted	Recovered from Well
A. Serum	1×10^3	270
B. Anti-mouse Ab + serum	2.6×10^3	450
Round 2 CFUs (from eluted phagemid-containing particles)		
	Pre-immune	Immune
A. Serum	1.6×10^4	2.7×10^4
B. Anti-mouse Ab + serum	4×10^4	3.8×10^4

Table 4.3. Recovery of phagemid from Experiment 3. CFUs correspond to stage F) of biopanning, indicated in Figure 4.1. Phagemid CFUs obtained after elution and retrieval of remaining bound particles in wells are indicated. The pG8SAET library was pre-adsorbed in TWELVE consecutive wells coated with pre-immune serum, or pre-immune serum bound to anti-mouse antibody, prior to biopannning against hyperimmune anti-*S.* Typhimurium serum in round 1. All other conditions correspond to those in Table 4.2.

e. Experiments 2-3: Discussion

In Experiments 2-3, the anti-mouse antibody/ mouse serum combination was compared to serum alone in the context of pre-absorption of the library in consecutive wells containing pre-immune serum. The goal for these experiments was to reduce the level of background binding to wells and serum components other than anti-*S.* Typhimurium antibodies. In a study that successfully identified four known antigens and one novel antigen of *Brugia malayi*, a similar pre-incubation strategy was used (Gnanasekar *et al.*, 2004). A 1:100 dilution of pooled serum from three different categories of pathology, each consisting of 10 non-immune individuals, was used to coat wells directly. The *B. malayi* cDNA phage display library was incubated consecutively in each well coated with a different category of non-immune serum prior to panning against a well coated with pooled serum from immune individuals. The same procedure was performed for each of four consecutive rounds of biopanning (Gnanasekar *et al.*, 2004).

Experiments 2-3 also include the recovery of phagemid particles not released by elution buffer through the addition of host cells to wells after elution. This method allowed the isolation of a clone encoding a particular binding domain of von Willebrand factor binding protein from a *Staphylococcus lugdunensis* library that was not obtained by elution with low pH (Nilsson *et al.*, 2004).

Experiments 2-3 revealed that the use of capturing antibody does not enhance the level of enrichment achieved by biopanning with serum alone. Additionally, consecutive pre-incubation of the library in 4 or even 12 wells containing pre-immune serum does not improve enrichment levels. Finally, these experiments indicated that a small percentage of phage not eluted in the first round constitute a

significant loss of enrichment in conventional biopanning, as demonstrated by the very large proportion of non-eluted phagemid in the second round.

f. Experiment 4: Results

The subsequent trials were performed without the use of capturing antibody. The library was pre-incubated with 10 µl or 20 µl of untreated or heat inactivated non-immune serum (obtained from non-immunised CBA/Ca mice other than those used for generation of hyperimmune *S.* Typhimurium serum) prior to biopanning against anti-*S.* Typhimurium serum. Non-immune serum was used instead of pre-immune serum in order to conserve pre-immune serum for future experiments. A single round of biopanning yielded phagemid as indicated in Table 4.4. Phagemid recovered from biopanning with library having been pre-incubated with 10 µl untreated non-immune serum were used in a second round of biopanning in Experiment 4. For this and subsequent biopannings, phagemid from eluted particles were combined with phagemid subsequently recovered from the corresponding well. The phage stock was pre-incubated with non-immune serum as it had been in the first round, after which it was added to a well containing non-immune (A) or anti-*S.* Typhimurium (B) serum. Phagemid recovered from round 2B were panned a third time against non-immune (A) or anti-*S.* Typhimurium (B) serum. Phagemid recovered from round 3B were panned a fourth time against non-immune (A) or anti-*S.* Typhimurium (B) serum. The resulting phagemid recoveries are indicated in Table 4.5.

Table 4.4. Phagemid recovery after one round of biopanning with particles having been pre-incubated in untreated or heat-treated non-immune serum. CFUs correspond to stage F) of biopanning, indicated in Figure 4.1. Serum was added to the library and incubated for 15 min. at RT prior to addition of the mixture to wells. *These phagemid were used for subsequent biopanning in Experiment 4.

Pre-incubation serum	CFUs eluted, recovered from well
*10 µl untreated	620, 130
10 µl heat-treated	570, 230
20 µl untreated	700, 130
20 µl heat-treated	640, 150

Ligand	Round 2 CFUs eluted, recovered from well	Round 3 CFUs eluted, recovered from well	Round 4 CFUs eluted, recovered from well
Non-immune serum	830, 270	420, 2×10^3	2.7×10^3, 8×10^3
Immune serum	740, 500	760, 2×10^3	5.1×10^3, 8×10^3

Table 4.5. Recovery of phagemid from Experiment 4. CFUs correspond to stage F) of biopanning, indicated in Figure 4.1. Phagemid recovered from the biopanning indicated in Table 4.4 were used in subsequent rounds of panning. Each phage preparation was pre-incubated with 10 µl of untreated non-immune serum prior to each round of panning. Propagated phage were panned sequentially against the same ligand.

g. Experiment 4: Discussion

Experiments were performed to assess differences in phagemid recovery after a single round of panning with library phage having been pre-incubated with 10 or 20 µl of untreated or heat-treated non-immune serum. Since complement is a serum component that may potentially damage phage particles, heat treatment was performed to inactivate it. However, no difference was observed among all four treatments. In Experiment 4, addition of non-immune serum to the library prior to biopanning was investigated in successive rounds of biopanning, with a parallel panning against pre-immune serum as a reference point. The multiple panning rounds revealed a strong enrichment for phagemid that could not be eluted. However, this enrichment was observed at the same level for both anti-*S.* Typhimurium serum

as well as pre-immune serum, which indicated that although the enrichment was strong, it was not likely to have resulted from antibodies directed against *S.* Typhimurium. In contrast, the addition of a non-immune antibody preparation to a random peptide phage display library prior to biopanning may have contributed to the successful isolation of epitopes specifically recognised by the antibodies of individuals exposed to *Borrelia burdorferi* (Kouzmitcheva *et al.*, 2001). Notably, the mixture was centrifuged to remove complexes prior to biopanning. However, many factors could have contributed to the success, since this study used purified antibodies that were also pre-adsobed against wild-type phage. Another study added non-specific antibody in solution with a random peptide library but did not remove the complexes prior to successfully panning for Equine herpesvirus type 1 epitopes (Birch-Machin *et al.*, 2000).

h. Experiment 5: Results

In the next several experiments (Experiment 5), the library was pre-incubated with non-immune serum, then was sequentially incubated in 7 wells coated with non-immune serum, prior to biopanning against anti-*S.* Typhimurium serum in the first round. In one of these experiments, about 5×10^3 phagemid were obtained by elution, and a further 1×10^3 were recovered from the well after elution in the first round. The combined progeny of the phagemid from this round were panned against non-immune and immune serum. This resulted in 1×10^4 and 6.6×10^4 eluted phagemid from wells coated with non-immune and immune serum, respectively. A

further 800 and 5×10^3 phagemid were recovered from the wells after elution, respectively. Several repetitions of this experiment could not reproduce the result.

i. Experiment 5: Discussion

In Experiment 5, addition of non-immune serum to the library prior to biopanning was combined with pre-absorption in wells coated with pre-immune serum. Several experiments were performed, one of which potentially exhibited a significant and specific enrichment. However, this experiment could not be repeated despite multiple attempts. Therefore, goals were set for subsequent experiments to optimise other biopanning parameters with the aim of improving the frequency in which potentially successful enrichments occur.

j. Experiments 6-8: Results

Next, different amounts of serum were used to coat wells in order to assess background binding of phage particles in Experiment 6. The anti-*S.* Typhimurium serum used throughout biopanning experiments (the final bleed from CBA mice) was determined to be at a concentration of 50 mg/ml as measured by Coomassie Plus Protein Assay Reagent (Pierce). Biopanning was performed following the original protocol established for the *S. aureus* pG8SAET library, but particles derived from the pG8SAET vector without inserted foreign DNA were used instead of the library. In Experiment 7, different blocking solutions were tested for their ability to reduce the level of background binding. This time, the library itself was used owing to the

fact that the wells were not coated with ligand prior to blocking. For Experiment 8, the anti-*S.* Typhimurium serum was divided into two fractions by precipitation with saturated ammonium sulphate. Both fractions were dialysed against PBS prior to coating in wells for biopanning. Wells were blocked with milk prior to addition of the library for biopanning. Phagemid recoveries (in CFUs: see stage F of biopanning indicated in figure 4.1) for the three experiments are shown in Table 4.6, 4.7 and 4.8.

Serum quantity	Eluted	Recovered from well
10 µl	1×10^3	160
*2 µl	1.6×10^3	240
0.2 µl	2.6×10^3	240
0.02 µl	4.2×10^3	380
0 µl	1.1×10^4	920

Table 4.6. CFU Phagemid recovery from Experiment 6. No-insert phage were used in mock panning instead of library phage. * Indicates quantity of serum normally used for coating in other experiments (0.1 mg protein).

Blocking solution	Eluted	Recovered from well
Fish gelatin	8×10^4	7×10^3
Milk	160	100
None	1.9×10^4	3.8×10^3

Table 4.7. CFU Phagemid recovery from Experiment 7. Wells were not coated prior to blocking in mock single-round biopanning. Library phage were used in this experiment.

Ligand	Eluted (% E-tag +)	Recovered from Well (% E-tag +)
ROUND 1		
0.1 mg supernatant fraction	370 (11%)	260 (12%)
0.1 mg IgG-enriched fraction	810 (10%)	330 (10%)
ROUND 2		
0.1 mg supernatant fraction	3×10^6 (21%)	N/C (36%)
0.1 mg IgG-enriched fraction	3×10^5 (33%)	N/C (51%)

Table 4.8. CFU Phagemid recovery from Experiment 8. Wells were coated with partially purified ligand as indicated, and were subsequently blocked with milk prior to biopanning with library phage. N/C: not counted. Proportion of phagemid expressing the E-tag are indicated in parentheses.

k. Experiments 6-8: Discussion

In Experiment 6, the binding capacity of the wells was tested by coating wells with different amounts of the anti-*S.* Typhimurium serum and observing the number of non-recombinant phagemid particles that bound after a single round of mock biopanning. Since non-recombinant phagemid particles do not display any foreign polypeptides, any binding must be non-specific. This experiment revealed that coating was maximal with the highest concentration of serum (10 µl), but the number of bound phagemid was not significantly lower than the typically used quantity (2 µl, which contains 0.1 mg protein), which was in turn not very much lower than for the well coated with 0.2 µl of serum.

In Experiment 7, different blocking solutions were tested for the ability to prevent binding of the library phage to uncoated wells. Up to this point, no blocking solution had been used during biopanning. This was because extensive washing with PBS-T had been sufficient for the positive control biopanning of the *S. aureus* library, so no blocking solution was used. Other successful studies also have not needed a blocking agent (Sheu *et al.*, 2003; Wall *et al.*, 2003). However, the total number of phagemid that bound in the first round of control biopanning the *S. aureus* library was much higher than had been the case for the *S.* Typhimurium library. Thus, the proportion of non-specifically binding phagemid was much lower for the *S. aureus* panning and therefore blocking was less important for that panning, and vice versa. Skimmed milk has been used as a blocking agent in successful biopanning procedures (Kjaer *et al.*, 1998; Theisen *et al.*, 2000). Fish gelatine has been evaluated among other blocking agents for quantitative detection of antigen (Kaur *et al.*, 2002). Biopanning the *S.* Typhimurium against empty wells blocked with the different

solutions yielded surprising results. An empty well blocked with fish gelatin caused more particles to bind than an unblocked empty well, implying that fish gelatine is "sticky" for M13 phage. This is significant because it shows that a poorly chosen blocking solution could complicate the interpretation of positive results. Since blocking with milk was shown to significantly reduce non-specific binding of the library to the wells, this step was added to subsequent pannings.

In Experiment 8, the mouse serum used in biopannings was partially purified by precipitation with saturated ammonium sulphate. This procedure was predicted to divide the serum into an IgG-enriched fraction (precipitate) and an IgG-depleted fraction (supernatant). Both of these fractions were dialysed against PBS prior to use in biopanning experiments. This is significant because some serum components having a very small molecular weight (<15 kDa) may have been lost during the procedure. Subsequent use of these two fractions for biopanning resulted in a high level of enrichment between the first and second rounds of biopanning for both fractions. Screening for E-tag expression further supported the probability that a successful enrichment had occurred. Importantly, this experiment did not contain a negative control owing to the fact that very little non-immune CBA serum was available, thus precluding a similar partitioning of this serum into IgG-enriched and IgG-depleted fractions.

l. Experiment 9: Results

In Experiment 9, a well was coated with 0.2 mg of the IgG-enriched fraction of anti-*S.* Typhimurium serum, blocked with milk, and used for biopanning the

library phage. All 2176 phagemid recovered after elution and subsequent recovery from the well were screened for E-tag expression. Phagemid with inconclusive E-tag expression were considered positive and were included in the total of 179 clones selected for further screening. Stationary phase cultures were made for each phagemid clone, and these were combined in equal volumes to form a mixed culture which was used for propagation of phage in soft agar on plates. Phage were extracted and used for a second round of biopanning, which yielded 1×10^5 eluted phagemid as well as a number of phagemid, recovered from the well after elution, that were not counted.

m. Experiment 9: Discussion

In Experiment 9, a well was coated with twice as much IgG-enriched fraction as was used for Experiment 8 (0.2 vs 0.1 mg). To ensure that no antigens were lost between the first and second rounds of biopanning, all 2176 colonies recovered from elution and after elution were screened for E-tag expression. Colonies with very weak and potentially negative results were included in the pool chosen for further analysis as a precautionary measure. Phage particles propagated on a stoichiometrically mixed culture of these clones were used in a second round of biopanning, which resulted in a strong enrichment for both eluted and non-eluted phage. The strong enrichments in Experiments 8 and 9 may have been due to a loss of one or more inhibitory components lost from the serum during dialysis. However, the specificity of the enrichments observed in these two experiments could not be ascertained due to a lack of negative control.

C. Results & Discussion: Screening

1. Results

a. Sequencing of clones and confirmation of E-tag expression

Plasmid DNA was isolated from each of the 179 clones isolated from Experiment 9 using a Wizard miniprep kit (Promega). Primers "etseq" and "etag" were used to sequence the DNA from the 5' and 3' side of insertion site, respectively. The sequencing primers annealed at a distance sufficiently far from the junction at both the 5' and 3' end of the insert to permit accurate assessment of the reading frame at both ends. Most sequencing reactions yielded data sufficient for identification of genes, their orientation and reading frames, and insert size. A reading frame of (+2) was required at the 5' end of the insert to form an in-frame fusion with the signal peptide. In other words, the first codon of the insert was formed by nucleotides 3-5. An in-frame fusion with the E-tag occurred at the 3' end when the last three nucleotides of the insert formed the last codon of the insert coding sequence (CDS). Data from successful sequencing reactions were used to perform BLAST searches to identify the sequences contained in each clone. The resulting data were then consolidated into Table 4.9. Additionally, colonies were screened once again for E-tag expression (Figure 4.3). TG1 harbouring pG8SAET with no insert was the negative control, while a pG8SAET construct containing the *S.* Typhimurium *rplL* gene (described below) served as the positive control.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Antigenicity	Typhi	gene/region	name of protein	comments
1	++++	Y	Y	43	(-3)	Y	1.51	100%	recJ	ssDNA exonuclease	5'-->3' specific, Mg dependent
2	+	Y	Y	149	(+3)	Y	1.99	100%	accB	acetylCoA carboxylase,	carrier of biotin
										BCCP subunit	
3	++++	Y	Y	217	N/A	Y	2.46	98%			sequence region between *corA* and *dbpA*
4	+		Y	>349	(+)	Y	1.81	99%	pduG	putative cobalamin	sequence quality not good at 5' junction
										adenosyltransferase	
5	-	Y	Y	31	(-2)	*	(-0.2)	96%	STM1697	putative diguanylate	(+2) contains 2 TAG's, (+1) and (+3) are ORFs
										cyclase/phosphodiesterase	
										domain 2	
6	++	Y	*	125	(+2)	*	2.06	N/A	orfF	hypothetical mobilization	non-conjugative *E. coli* plasmid RSF1010;
										operon protein F	base #309 (just before insert) missing
											from vector; (+2) contains one stop codon;
											(+1) & (+3) contain 1 'TAG' each
7	-	Y	Y	374	(+3)	Y	1.28	N/A	STM2697	putative tail protein	Fels-2 prophage: similar to
											orfG protein in phage 186
8	-	Y	Y	86	(+2)	Y	2.27	N/A	ssbB	single-stranded DNA	pSLT
										binding protein	
9	++++	Y	Y	97	(+2)	Y	2.26	97%	cysA	sulfate transport ATP-binding protein	
10	-	Y	Y	149	(+3)	Y	1.95	98%	dxr	1-deoxy-D-xylulose	non-mevalonate pathway of
										5-phosphate	isoprenoid biosynthesis
										reductoisomerase	
11	-	Y	*	90	(+3)	*	1.15	100%	yfcY	paral putative acetyl-	putative 3-ketoacyl-CoA
										CoA acetyltransferase	thiolase in *S.* Typhi
12	-	Y	*	67	(-2)	*	0.06	N/A	repB/mobA	putative primase/	non-conjugative
										replication protein/	*E. coli* plasmid RSF1010
										mobilization protein	
13	-	N	N	>334	(-1)	unknown	N/A	96%	avtA	valine-pyruvate	no CDS; full of stop codons
										aminotransferase	

Table 4.9 (continued on following pages). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
14	-	N	Y	>870	(+1;-)	unknown	2.63	N/A	oafA; ygaP	O-antigen acetylase;	fusion of 2 different DNA fragments
										putative rhodanese-	
										related sulfurtransferase	
15	-	Y	Y	53	(-2)	Y	2.57	100%	glpA	anaerobic sn-glycerol-3-phosphate	
										dehydrogenase, large subunit	
16	+++++	Y	N	238	N/A	N	N/A	99%	ybgS		full of stop codons
17	+	Y	Y	744	(+1)	Y	2.32	98%	ansA	cytoplasmic L-asparaginase I	
18	-	Y	N	436	(-3)	N	N/A	99%	glpA	anaerobic sn-glycerol-	(+1) & (+3) frames each
										3-phosphate	contain one stop codon
										dehydrogenase, large subunit	
19	-	Y	Y	218	(-1)	Y	1.62	94%	fliC	phase 1 flagellin	first 40 amino acids of ORF have low-level
											homology with an *E. coli* flagellin subunit
20	-	Y	Y	199	(-1)	N	1.43	97%	STM3025	putative cytoplasmic	transcriptional regulator sprB
										protein	
21	-	Y	Y	166	(-3)	N	2.78	99%	lysP	lysine-specific permease	APC family
22	++									DELETION MUTANT	
23	-	Y	N	364	(+1)	N	N/A	98%	ddlA	D-alanine-D-alanine	full of stop codons
										ligase A	
24	+	Y	Y	~880	(+2)	Y	2.29	99%	STM2722	probable terminase	Fels-2 prophage: similar to gpP,
										subunit	ATP charging, in phage P2
25	+++++	Y	Y	157	(+2)	Y	2.67	98%	STM3167	putative diadenosine tetraphosphate (Ap4A) hydrolase	
26	+	Y	Y	587	(+3)	Y	2.45	98%	tdcB	catabolic threonine	induced anaerobically when aa's
										dehydratase	are plentiful and glucose is absent
27	+	Y	Y	143	(+3)	Y	2.08	97%	yfeK	putative periplasmic protein	
28	-										poor growth; sequence not obtained
29	-	Y	N	496	(+1)	N	N/A	98%	ddg	cold shock-induced	full of stop codons
										palmitoleoyl transferase	
30	-									DELETION MUTANT	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
31	-	Y	Y	63	(+1)	Y	0.59	100%	trmE	GTPase for tRNA modification and thiophene and furan oxidation	
32	+	Y	Y	201	(+1)	Y	2.77	N/A	sodCI	Cu, Zn superoxide	Gifsy-2 prophage
										dismutase	
33	-	Y	Y	228	(-2)	Y	2.35	98%	serB	3-phosphoserine phosphatase	
34	+	Y		373	(+2)	Y	2.37	100%	deaD	ATP-dependent RNA	CDS begins halfway through
										helicase/cysteine	insert sequence; acid-induced
										sulfinate desulfinase	
35	-	Y	Y	~470	(+)	Y	2.94	98%	metG	methionine tRNA	sequence quality
										synthetase	not good at 5' junction
36	-	Y	Y	669	(+1)	Y	2.60	66%	rci	shuffion-specific	CDS is preceded by 1 stop codon
										recombinase	& begins halfway through the insert
											sequence; poor growth
37	-	Y	Y	203	(+3)	Y	2.14	99%	edd	6-phosphogluconate dehydratase	
38	-	Y	Y	122	(+3)	Y	2.60	100%	nagE	PTS system; N-acetyl-	CDS begins 2/3 of the way
										glucosamine-specific	through the insert
										enzyme IIABC	
39	-	Y	Y	98	(+3)	Y	1.73	96%	pyrF	orotidine-5'-phosphate decarboxylase	
40	+	Y	Y	519	(+1)	Y	2.66	59%	ybeQ	putative TPR repeat	CDS is preceded by 1 stop codon
										protein	& starts halfway through insert sequence
41	+	Y	Y	135	(+1)	Y	1.11	100%	kdsA	3-deoxy-D-manno-octulosonic acid 8-P synthetase	
42	-	N	Y	>519	(+;+3)	N	2.47	N/A	res; ylaB	restriction-modification	insert is a fusion of 2 fragments
										enzyme; putative diguanylate	
										cyclase/phosphodiesterase	
43	-	Y	Y	143	(-1)	Y	2.64	98%	yjiY	putative carbon starvation protein	
44	+	Y	N	79	(-1)	N	N/A	100%	ftsN	essential cell division	each of the 3 possible reading frames
										protein	contains 1 stop codon
45	++									DELETION MUTANT	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
46	+++	N	N	>541	(-2)	N	N/A	97%	yqgA	putative inner membrane	full of stop codons
										transport protein	
47	-	Y	N	128	(-3)	N	N/A	N/A	orf46	putative integrase	*S.* Choleraesuis 50k virulence plasmid;
										protein	each reading frame contains 1 stop codon
48	-	Y	Y	200	(+3)	Y	2.59	100%	ybeX	Mg and Co efflux protein	CDS is preceded by 1 stop codon &
											begins 27 bases into the insert; haemolysin-
											related protein; contains CBS domain
49	-	Y	N	303	(-3;+1)	Y	N/A	N/A	yfhP; adiY	believed to be involved in	contains 1 stop codon;
										assembly of Fe-S	insert is a fusion of 2 DNA fragments
										clusters; transcriptional activator of adiA	
50	+	Y	N	506	(+1;+3)	Y	N/A	99%	yiaN; yiaO	putative DedA family; putative dicarboxylate-binding protein	
51	-	Y	N	205	(+1;+2)	Y	N/A	97%	STM0610;	putative component of anaerobic dehydrogenases;	
									STM0611	putative oxidoreductase protein	
52	-	Y	Y	390	(+1)	Y	2.30	100%	lonH	putative ATP-dependent	CDS is preceded by 2 stop codons &
										protease	starts 1/3 way through insert sequence
53	++++	Y	Y	127	(+2)	Y	2.38	99%	ytfE	putative cell morphogenesis protein	
54	-	Y	Y	35	(-2)	Y	1.68	N/A	STM4489		putative superfamily I DNA helicase
55	+	Y	N	405	(+1;-2)	Y	N/A	99%	hpt; gcd	hypoxanthine phosphoribosyltransferase; glucose dehydrogenase	
56	++	Y	N	267	N/A	N	N/A	97%			RSA repetitive element; full of stop codons
57	-	N	Y	>145	(-1)	unknown	2.15	95%	ybjF	putative tRNA (uracil-5-)-methyltransferase	
58	-	Y	N	328	(+2)	N	N/A	N/A	PSLT036	putative transposase,	pSLT; full of stop codons
										IS200-like	
59	-	Y	N	151	(-3)	N	N/A	N/A	9	tailspike protein	P22; full of stop codons
60	+	Y	*	182	(-2)	*	2.56	96%	sciP	putative outer	ompA family; contains 2 TAG's
										membrane protein	
61	+	N	N	>250	(-1)	N	N/A	95%	STM2010	putative cytoplasmic	full of stop codons
										protein	
62	+++	Y	Y	131	(-1)	Y	0.88	97%	nrdD	anaerobic ribonucleoside-triphosphate reductase	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
63	+	Y	Y	464	(+3)	Y	2.21	98%	tas	putative aldo/keto reductase	
64	-	Y	Y	233	(+3)	Y	2.53	99%	deaD	ATP-dependent RNA helicase/ cysteine sulfinate desulfinase	
65	-	N	N	>304	(-2)	unknown	N/A	N/A	STM1041	probable minor tail	Gifsy-2 prophage
										protein	
66	-	Y	Y	687	(+1)	Y	2.22	<65%	STM0351	putative cation efflux	Paralog of *E. coli* integral transmembrane
										system protein	protein; acridine resistance
67	-	Y	Y	206	(+3)	Y	0.83	100%	dnaX	DNA polymerase III	CDS is preceded by 1 stop codon &
										gamma subunit	starts 2/3 way through insert sequence
68	+	Y	Y	540	(+1)	Y	2.62	98%	STM4305	putative anaerobic dimethyl sulfoxide reductase, subunit A	
69	+	Y	N	363	(+1)	Y	N/A	N/A	stbA		*E. coli* plasmid pCoo
70	-	N	N	>450	(-3)	unknown	N/A	99%	engA	putative GTP-binding	full of stop codons
										protein	
71	+	Y	Y	483	(+1)	Y	2.3	99%	kdsB	CTP:CMP-3-deoxy-D-manno-octulosonate transferase	
72	-	N	N	>157	(-3)	unknown	N/A	98%	mtlA		PTS family, mannitol-specific enzyme
											IIABC components; contains 1 stop codon
73	-	N	N	>454	(-1)	unknown	N/A	98%	phoH		PhoB-dependent, ATP-binding *pho* regulon
											component; full of stop codons
74	-	Y	N	415	(-3)	N	N/A	N/A	traA	fimbrial subunit	conjugative transfer; contains 1 stop codon
75	-	Y	Y	86	(-1)	Y	2.73	98%	cgs	cystathione gamma-synthase	
76	-	Y	Y	515	(+1;-3)	N	1.92	N/A	STM1537;	putative Ni/Fe-hydro-	insert sequence is a fusion of
									int	genase 1 b-type cyto-	2 DNA fragments
										chrome subunit; P22 integrase	
77	-	Y	Y	44	(+1)	N	1.38	100%	holB	DNA polymerase III, delta prime subunit	
78	-	Y	N	133	(-2)	*	2.49	100%	folB	dihydroneopterin	also has dihydoneopterin triphosphate
										aldolase	2'-epimerase activity; contains 1 TAG
79	-	N	Y	>107	(-1)	unknown	2.33	98%	eutG	paral putative transport protein in ethanolamine utilization	
80	-	N	N	>247	(-1)	unknown	N/A	98%	btuB	outer membrane	contains 1 stop codon
										receptor for transport of vitamin B12, E colicins, and bacteriophage BF23	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
81	+	N	N	>268	N/A	N	N/A	100%	sohB/yciK		intergenic region; full of stop codons
82	-	Y	Y	369	(+1)	Y	2.62	99%	STM4551	putative diguanylate cyclase/phosphodiesterase domain 1	
83	-	N	Y	>105	(-2)	unknown	2.67	100%	orf2	proline/threonine-rich	*S.* Typhi; 3' of *smpB*
										protein	
84	-	N	N	>383	(-)	N	N/A	N/A	STM1025	Gifsy-2 prophage	contains several stop codons
										putative lipoprotein	in all forward frames
85	++									DELETION MUTANT	Bases 283-309 (inclusive) deleted
86	-	Y	N	89	(+1)	N	N/A	N/A	orf56		very small P22 protein; contains 1 stop codon
87	+	Y	N	160	(+2)	Y	0.74	N/A	traR		1 stop codon precedes the CDS;
											overlaps with clone 101 insert
88	-	N	Y	>125	(+)	N	2.10		Z1868	putative replication protein O of prophage CP-933X	
										in enterohemmoragic *E. coli* O157:H7	
89	-	Y	Y	242	(+3)	Y	2.60	100%	ycdX	putative histidinol	hydrolase of the PHP family
										phosphatase	
90	++	Y	Y	143	(+3)	Y	2.54	97%	nrfG		part of formate-dependent nitrite reductase
											complex; involved in attachment of haem c
											to cytochrome c552
91	-	Y	N	172	(+3)	N	N/A	99%	flhA	flagellar biosynthesis; possible export of flagellar proteins	
92	+++++	Y	Y	59	(-2)	Y	unknown	?	gpsA	glycerol-3-phosphate dehydrogenase (NAD+)	
93	-	N	Y	>282	(+)	Y	2.72	98%	STM4306	putative anaerobic dimethyl sulfoxide reductase, subunit B	
94	+	Y	N	175	(+2)	Y	N/A	100%	nusA	transcription pausing;	RBS and first 6 amino acids
										N utilization	
95	-	Y	Y	145	(-3)	Y	1.73	97%	yigA/orf235	putative cytoplasmic	(+3) frame contains one 'TAG'
										protein	
96	-	Y	N	381	(+3;+1)	Y	N/A	96%	yfgL; engA	putative serine/threonine protein kinase; putative GTP-binding protein	
97	-	Y	Y	73	(-2)	*	1.22	98%	ccmE	periplasmic haeme-dependent peroxidase;	
										cytochrome c-type biogenesis protein	
98	-	Y	Y	28	(-3)	*	0.10	100%	yijD	putative inner membrane protein	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
99	+	N	N	>429	(-3)	unknown	N/A	99%	nlpI	cell division lipoprotein	contains 1 stop codon;
											located immediately after deaD gene
100	+	Y	Y	128	(+3)	Y	2.49	75%	pilV	shufflon protein A;	plasmid R64; IncI1 plasmid
										type IV prepilin	
101	++++	Y	Y	181	(+2)	Y	0.74	N/A	traR		2 stop codons precede the CDS;
											overlaps with clone 87 insert
102	-	Y	N	220	(+1)	N	N/A	99%	flgM	anti-sigma 28 factor	negative regulator of flagellin synthesis;
											contains 1 stop codon near end of insert
103	-	N	Y	>384	(+1)	unknown	2.52	98%	orf2	proline/threonine-rich	3' of *smpB* in *S.* Typhi clinical isolate
										protein	
104	+	Y	Y	112	(-3)	Y	0.80	100%	fimD	outer membrane usher protein	
105	-	Y	Y	507	(+1)	Y	2.44	97%	eutE	aldehyde oxidoreductase	ethanolamine utilization protein
106	+++									DELETION MUTANT	
107	++	Y	N	86	(-2)	N	N/A	90%	yafS		putative SAM-dependent methyltransferase
108	-	Y	Y	96	(+1)	Y	0.46	97%	ybgJ	putative carboxylase	
109	+++			>45							(sequence not available)
110	-	Y	Y	89	(+3)	Y	0.78	100%	focA	probable formate transporter	
111	-	N	N	>380	(-1)	unknown	N/A	100%	STM2475	putative cytoplasmic	very small protein
										protein	
112	-	Y	N	215	N/A	N	N/A	98%	pagO		no CDS: intergenic region full of stop codons
113	+	Y	Y	70	(+2)	Y	0.77	98%	ptsN	sugar specific PTS family, enzyme IIA, also regulates N metabolism	
114	++++	Y	Y	124	(+2)	Y	1.30	97%	oat	putative acetylornithine aminotransferase	
115	++	Y		331	(+2)	Y	2.18	99%	rsmC	16S rRNA m2G 1207	contains 1 stop codon at beginning of insert
										methylase	
116	-	Y	Y	192	(+1)	Y	1.27	N/A	1	P22 portal protein	similar to phage APSE-1 P19
117	+	Y	*	89	(-2)	*	2.52	N/A	9	bifunctional P22 protein:	(+1) frame contains one 'TAG'
										tailspike & endorhamnidase	
118	+++++	Y	Y	118	(+2)	Y	2.24	98%	yhjJ	putative Zn-dependent peptidase	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
119	-	Y	N	251	(+3)	Y	1.96	98%	ydcP	putative collagenase	CDS begins 90 bases into insert
120	+	N	Y	>107	(+)	Y	1.22	100%	lpxK	tetraacyldisaccharide	Lipid A 4'-kinase
										4'-kinase	
121	+	N	?	>277	(+1;+)	Y	1.65	N/A	so667; mgs	putative collagenase;	may be a fusion of 2 fragments;
										methylglyoxal synthase	central junction unsequenced
122	+	Y	Y	244	(+2)	Y	2.58	N/A	16	P22 DNA transfer	similar to phage APSE-1 P35
										protein (injection protein)	
123	+	N	Y	>122	(+)	Y	0.73	99%	flgN		export chaperone involved in
											flagellar biosynthesis
124	-	Y	Y	74	(-2)	Y	0.41	71%	STM4534	putative NtrC family	Paralog of *E. coli psp* operon
										transcriptional	transcriptional activator
										regulators, ATPase domain	
125	-	Y	Y	165	(+1)	Y	1.52	98%	pykA	pyruvate kinase II, glucose stimulated	
126	++	Y	Y	109	(+2)	Y	0.93	99%	yijO	hypothetical transcrip-	AraC/XylS family
										tional regulator	
127	+	N	Y	>296	(+)	Y	2.09	N/A	STM1055	hypothetical protein	Gifsy-2 prophage
128	+	N	Y	>75	(+)	unknown	unknown	?	ratB	putative outer membrane protein	
129	++	N	N	>87	N/A	N	N/A	<66%	STM4422/	no CDS: intergenic region	
									STM4423		
130	++	Y	Y	276	(+1)	Y	0.91	100%	artI	arginine-binding protein	ABC superfamily
131	+	N									sequence not obtained
132	-	N	N	>154	(-3)	N	N/A	99%	rfbG	CDP glucose 4,6-dehydratase; LPS side chain defect	
133	-	Y	N	278	(N/A;	N	N/A	N/A	STM2769/	repressor of phase 1	insert is a fusion of 2 fragments
					+3)				fljA; entB	flagellin; 2,3-dihydro-	
										2,3-dihydroxybenzoate synthetase, isochorismatase	
134	+++	Y	Y	88	(+2)	Y	2.23	98%	ydiY	putative salt-induced and acid-induced OMP	
135	-	Y	Y	336	(+1)	Y	2.13	99%	yheT	putative hydrolase	
136	-	Y	Y	72	(+1)	Y	(-0.01)	97%	yhhS	putative MFS family transport protein	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
137	-	Y	Y	218	(+1)	Y	1.98	100%	ydfG	putative oxidoreductase	
138	-	Y	Y	251	(+3)	Y	2.33	N/A	traU	nucleotide-binding	plasmid R64
										protein	
139	++++	Y	Y	70	(-1)	Y	0.18	98%	STM3356	putative cation transporter	
140	-	N	Y	>59	(+3)	unknown	unknown	?	shdA	similar to the C-terminal	very large protein; subspecies I specific;
										region of AIDA; IcsA;	Peyer's patch colonization & shedding factor
										putative cation transporter	
141	-	N	Y	>115	(-2)	unknown	2.70	98%	pta	phosphotransacetylase	
142	-	N	Y	>96	(+2)	unknown	(-0.175)	97%	yccF	putative inner membrane prote	+1 is an ORF, but +2 has
										protein	3 stop codons before the CDS begins
143	+	N	N	>210	N/A	unknown	N/A	98%	yjfR/sgaT		intergenic region; full of stop codons
144	+	N	Y	>119	(+3)	unknown	unknown	?	miaB		methylthiolation of isopentenylated
											A37 derivatives in rRNA
145	-	N	N	>39	(-1)	unknown	unknown	?	ydbH	putative periplasmic protein	
146	-										sequence not obtained
147	+++	N	Y	>124	(+3)	unknown	1.51	99%	proX	glycine/betaine/proline	ABC superfamily
										transport protein	
148	++++	Y	Y	133	(+2)	Y	2.25	N/A	STM2235	putative phage protein	
149	++++	N	N	>63	N/A	unknown	N/A	100%	mfd		intergenic region; full of stop codons
150	-	N	N	>186	N/A	unknown	N/A	98%	nifJ/ynaF	intergenic region	adjacent to *dbpA* gene on *S.* Typhimuruim
											genome; opposite direction of clone 3
151	-	N	Y	>78	(+1)	unknown	(-0.5)	100%	wzx	flippase	LPS biosynthesis
152	-	Y	Y	99	(+3)	N	0.73	96%	mrcA	transpeptidase of	peptidoglycan synthetase
										penicillin-binding protein 1A	
153	+++	N	Y	>49	(-2)	unknown	(-0.08)	91%	STM2527	putative polyferredoxin	paralog of *E. coli* hydrogenase 4 Fe-S subunit
154	-	N	Y	>86	(+1)	unknown	unknown	N/A	traJ	nucleotide-binding	plasmid R64
										protein	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

Clone	E-tag	complete	ORF	size (bp)	frame	E-tag fusion	Anitgenicity	Typhi	gene	name of protein	comments
155	-	N	Y	>54	(+3)	unknown	unknown	?	pmrF	putative glycosyl transferase	LPS modification protein
156	++	N	Y	>82	(+1)	unknown	0.78	<60%	STM4497	putative cytoplasmic protein	
157	++										sequence not obtained
158	+++	N	N	>319	N/A	unknown	N/A	98%	pduB		intergenic region; full of stop codons
159	++++	N	Y	>56	(+1)	unknown	unknown	?	ycgA/yddA/yciA		plasmid CollB-P9; plasmid F
160	++	N	Y	>71	(-1)	unknown	2.20	N/A	PSLT047	putative cytoplasmic	pSLT
										protein	
161	+++	Y	Y	79	(-2)	Y	2.23	100%	ydeY	putative sugar transport	ABC superfamily
										protein	
162	-	N	Y	>76	(-2)	unknown	0.12	97%	yjiN	putative inner membrane protein	
163	+	N	Y	>85	(-1)	unknown	2.37	98%	ybiV(1)	putative hydrolase	HAD superfamily
164	+	N									sequence not obtained
165	+	N									sequence not obtained
166	++	N									sequence not obtained
167	-	N									sequence not obtained
168	-	N									sequence not obtained
169	-	N	Y	>150	(+1)	unknown	0.18	100%	glpG		
170	+++++									DELETION MUTANT	
171	+++++	Y	Y	118	(-1)	Y	0.90	97%	wcaL	putative glycosyl transferase	colanic acid biosynthesis
172	+++										sequence not obtained
173	-	Y	N	45	(+)	N	N/A	93%	STM0081		ribosomal binding site for STM0081
174	+++++	Y	N	329	(+1)	N	N/A	99%	ybbO	putative oxidoreductase	4 stop codons follow the CDS
175	-	Y	N	167	(-2)	N	N/A	98%	STM2357	putative amino acid	full of stop codons
										transporter	
176	-	N	Y	>70	(+3)	unknown	2.17	98%	STM1493	putative periplasmic protein; transporter; insert includes gene's stop codon	
177	-										sequence not obtained
178	+										sequence not obtained
179	+	N	Y	>201	(+3)	unknown	2.30	95%	STM0033	putative secreted 5'-nucleotidase	

Table 4.9 (continued from previous page). The 179 clones selected from Experiment 9 for further analysis. Bold numbers in column 1 indicate clones that were chosen for screening. Rows with bold borders indicate clones that were chosen for sub-cloning. E-tag expression levels are relative to the positive and negative controls. Column 3 indicates whether the entire insert sequence was obtained. Column 4 indicates whether the insert sequence constitutes a continuous open reading frame (ORF). A correct fusion to the signal sequence has a reading frame of (+2). * Indicates that an ORF in frame with the E-tag having one or more amber (TAG) stop codons. Column 8 contains the mean antigenic index (Jameson-Wolf, using Protean software from DNASTAR) of the 6 consecutive amino acids (indicated in bold in Appendix) with the highest antigenicity in the available sequence. Column 9 indicates amino acid identity with *S.* Typhi CT18. Plasmid sequences were not compared.

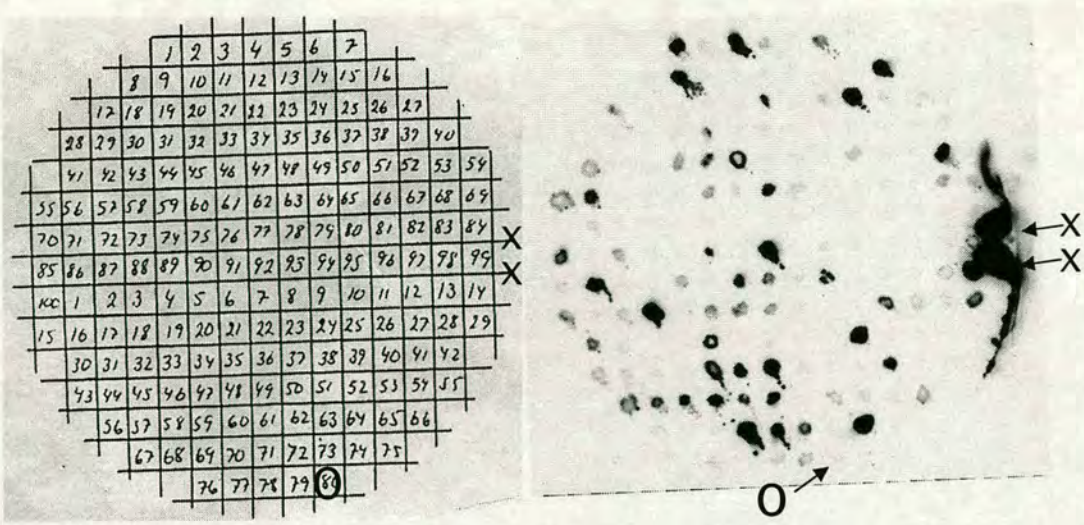


Figure 4.3. Re-screening of 179 clones from Experiment 9 for E-tag expression. Colonies of TG1 cells with pG8SAET constructs were grown on nitrocellulose, lysed, and probed with a monoclonal anti-E tag antibody. HRP-labelled secondary antibody was detected by chemiluminescence and exposed to X-ray film. The location of the positive control clone is marked with X, while the negative control is marked with O. All other numbers correspond to the number assigned to the clone tested.

b. Construction of positive control for screening

The *S.* Typhimurium *rplL* gene encodes the L7/L12 ribosomal subunit protein. This protein was identified as an antigen by the proteomic method (M. Mogensen, personal communication). Briefly, *S.* Typhimurium whole cell lysate was subjected to 2-D gel electrophoresis and subsequently analysed by Western blot using the final bleed BALB/c serum described in Chapter 3. Antigenic spots were excised from corresponding positions in a parallel 2-D gel and analysed by mass-spectrometry (data not shown). The protein having the smallest molecular weight, L7/L12, was chosen as the positive control for screening antigens in this thesis.

The *S.* Typhimurium *rplL* gene was cloned into pG8SAET, in frame with the signal sequence at the 5' end and with the E-tag at the 3' end. Briefly, primers "ribfor" and "ribrev" were used to amplify the *rplL* gene from *S.* Typhimurium SL1344 DNA. For blunt-end ligation, the PCR product was phosphorylated with T4

polynucleotide kinase and combined with vector DNA having been digested with *Sna*BI and dephosphorylated. The ligated DNA was then transformed into competent TG1 host cells, after which colony PCR was performed using primers "sasekv" and "etag." After analysis of PCR products by agarose gel electrophoresis (Figure 4.4), colonies were screened for E-tag expression. Plasmid DNA from an E-tag positive clone was then purified and sequenced using the primer "etag" to confirm that the *rplL* gene had been cloned correctly and that it contained no mutations.

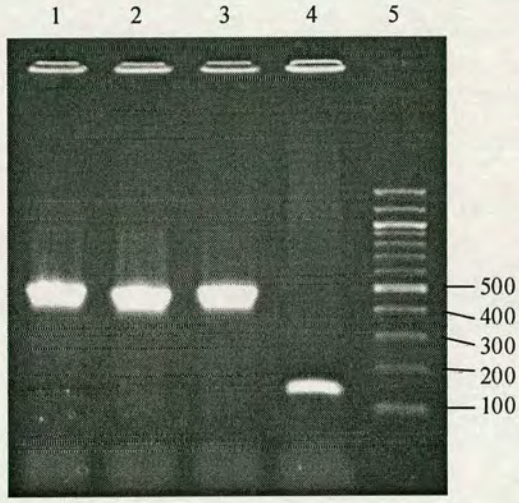


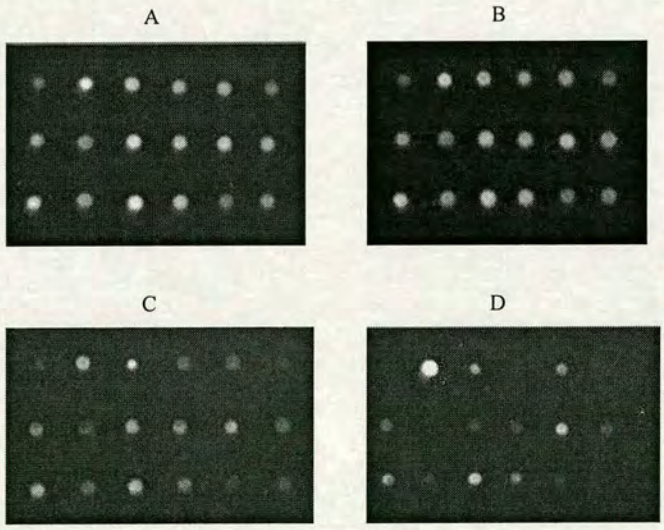
Figure 4.4. PCR of colonies resulting from transformation of the *rplL*/pG8SAET ligation. Primers "sasekv" and "etag" produce a band of approximately 150 bp (lane 4) when used to amplify pG8SAET with no insert. Lanes 1-3 are PCR products from independent colonies likely containing the *rplL* gene in pG8SAET. Lane 5 is the 100 bp ladder used to estimate DNA band sizes.

c. Screening of selected clones for antigenicity

All clones having a + orientation of the inserted DNA sequence (regardless of reading frame), as well as clones having no sequence data, were screened for antigenicity. Each of the clones to be screened, as well as the positive control and no-insert negative control, were grown in 1.5 ml tubes as 0.5 ml liquid cultures infected with helper phage to generate phage particles. The cultures were then centrifuged to remove cells and debris, and the supernatants were spotted onto nitrocellulose in 1 µl portions. The membranes were then blocked and subsequently probed with the same

serum used for biopanning (day17pc3) or with mouse monoclonal anti-E tag antibody. HRP-labelled secondary antibody was detected by chemiluminescence.

Different blocking solutions were compared during screening for antigens. All membranes were blocked in (A) 5% milk for an hour, and some were blocked in (B) 5% milk + 20% foetal bovine serum or (C) 5% milk with 10 % bovine serum for an additional hour. After testing the different blocking procedures, (B) was considered most suitable and was used for the majority of antigenicity screening. Phage lysates used in these experiments were tested for consistency of titre and were found to generate approximately 10^9 particles per ml. Each clone was screened at least twice from independent lysates, and the final 10 clones chosen for further analysis were screened four times. Dot blots of the final 10 clones (indicated in bold) are shown after three different blocking procedures in Figure 4.5.



E

-	+	**9**	11	**25**	50
53	67	**113**	**114**	**118**	**122**
126	130	**134**	**148**	158	X

Figure 4.5. Dot blots of phage lysates. Dot blots were probed with anti-*S.* Typhimurium serum (A-C) or with anti-E tag antibody (D). (A) and (D) were blocked with milk alone, while (B) was further blocked with foetal bovine serum and (C) was blocked with bovine serum. Positions of the negative and positive controls, as well as specific clones from Table 4.9, are indicated in (E). "X" indicates an error and should be disregarded. Numbers in bold indicate the 10 clones chosen for further analysis in the next chapter.

2. Discussion

a. Summary

In order to select candidate *S.* Typhimurium antigens from the pG8SAET library, it was panned against hyperimmune anti-*S.* Typhimurium serum obtained from CBA/Ca mice. Several parameters of biopanning were assessed, including the use of capturing antibody, depletion of the library with non-immune serum, blocking, multiple rounds, post-elution harvesting of phagemid from wells, and fractionation of ligand from the serum. After an inferred successful biopanning result, individual phage lysates were screened at least twice for possible binding of antibody from the hyperimmune serum. During this screening process, three different blocking protocols were compared for reduction of the signal-to-noise ratio between the constructed positive control clone and the negative having no displayed peptides. The screening process identified ten possible antigens displayed on the surface of phage, encoded by fragments of *cysA*, *STM3167*, *ytfE*, *ptsN*, *oat*, *gp16*, *yijO*, *STM2235*, *ydiY*, and *yhjJ*.

b. Analysis of Table 4.9

Phagemid DNA from all 179 clones chosen for further analysis was isolated and used to perform sequencing reactions. Data from successful sequencing reactions was used to perform BLAST searches, and results were compiled in Table 4.9. The results for re-screening for E-tag expression are shown in Figure 4.3 and included in

Table 4.9. Since colonies with potentially negative results were included, it is not surprising that some clones are actually negative for E-tag expression.

Interestingly, some of the clones are deletion mutants that contain no insert DNA but correct the disparity in reading frames between the signal peptide and E-tag. It is not clear at which stage these deletions occurred. However, clone 30 was identified as having the entire E-tag sequence deleted as shown by sequencing with the 5' primer. Since this clone was initially positive for E-tag expression (weakly to moderately so; data not shown), the deletion must have occurred during propagation after the first screening. It is possible that some unsuccessful sequencing reactions may be due to mutations in the regions of pG8SAET to which the sequencing primers anneal. A few clones contain insert fragments that are the result of the fusion of two different segments of DNA from *S.* Typhimrium DNA. This indicates that the ligation ratio of insert to vector should not be higher than what was used in the construction of this library in order to avoid this phenomenon.

For the clones in Table 4.9 with sufficient sequence data available, about 60% contain an insert in the plus orientation, which is only slightly higher than would be expected from a random screen. The average insert size of these clones is difficult to ascertain since the sequence data are incomplete. However, the clones for which some data are available have an average insert size of about 200 bases, which is slightly smaller than the average of 350 bases for the library. This is consistent with the observation that most biopannings result in an enrichment of clones having smaller inserts (Wilson and Finlay, 1998).

Table 4.9 includes a comparison of specific chromosomal *S.* Typhimurium sequences with those of the highly virulent human pathogen, *S.* Typhi CT18. The vast majority of sequences have a high identity with the *S.* Typhi chromosomal

sequences, as would be expected since the two strains are members of the same species. This fact supports the use of *S.* Typhimurium for the initial screening and identification of antigens that may be suitable for use in human vaccinations against *S.* Typhi.

Many peptide sequences possibly expressed by the clones in Table 4.9 are predicted to contain potentially antigenic regions, according to the Jameson-Wolf calculation for antigenicity prediction (Jameson and Wolf, 1988) performed with Protean software from DNASTAR. This calculation combines predictions of hydrophilicity, surface probability, flexibility, and secondary structure to produce an antigenic index. The corresponding specific regions, composed of six consecutive amino acids, are represented in bold type for each clone in the Appendix. The mean antigenicity for each specific region is represented in Table 4.9. Values greater than zero indicate possibly antigenic regions. Since an antigenic peptide can be very short in length, the possibility exists that short sequences from otherwise "missense" polypeptides encoded by many of the 179 clones could cross-react with antigenic sequences of *Salmonella* proteins. This possibility could explain the broad range of genes isolated after biopanning. Furthermore, peptide mimics of carbohydrate antigens may be selected through biopanning. Indeed, a peptide phage library was used to identify *S.* Typhi Vi polysaccharide epitope mimics through biopanning (Tang *et al.*, 2003).

Four loci are represented by more than one clone in table 4.9. An intergenic region adjacent to the *dbpA* gene is contained in clones 3 and 150, in opposite orientations. Although this region does not appear to encode any protein, the ORF in clone 3 is well expressed in the TG1 host cells, as evidenced by the strong level of E-tag expression. The *deaD* gene is present in the plus orientation in the insert of

clones 34 and 64, and clone 99 contains an insert from the chromosomal region immediately adjacent to *deaD*. However, none of the sequences overlap. The *glpA* gene is present in the inserts of clones 15 and 18. However, both are in the minus orientation and do not express the E-tag, which means this is likely to be a coincidence. On the other hand, the *traR* gene is present in the plus orientation in clones 87 and 101, whose insert sequences overlap. Interestingly, although both contain non-amber stop codons in the 5' region of the insert, both express the E-tag. This indicates that translation occurs by a non-conventional mechanism in these clones. Independently isolated clones with insert sequences that overlap have been characterised as containing a ligand binding region (Jacobsson and Frykberg, 2001). However, the *traR* gene is plasmid-borne; hence it is present in multiple copies (McClelland *et al.*, 2001). Therefore, it is possible that these two clones have been isolated by chance and not because of specific enrichment.

The insert of clone 8 is an uninterrupted ORF that is fused in the correct orientation and correct reading frames at both ends. Yet E-tag expression from this clone was not detected by screening with the monoclonal antibody. In contrast, clone 174 has an unexpectedly high level of E-tag expression, despite the fact that the ORF of the insert is followed by several non-amber stop codons and is in a different reading frame from the E-tag sequence. The mechanism governing translation in this clone cannot be explained at present.

Interestingly, clone 36, which grows poorly, encodes a shufflon-specific recombinase. The function of this protein has been described in Chapter 1 as regulating the expression of pili mediating self-association of *S.* Typhi. If the portion encoded within clone 36 produces enough active enzyme to disrupt *E. coli* cellular processes, it could explain the clone's poor growth. This clone was able to grow well

enough to obtain a sufficient amount of plasmid DNA for sequencing, which was not the case for clone 28. This highlights an inherent problem, the need for the host cell to survive and reproduce while expressing the recombinant fusions introduced. Therefore, any antigens that interfere strongly with host cell homeostasis or reproduction will not be recovered from this type of screening.

The only known *Salmonella* antigen among the 179 clones is the flagellin subunit encoded by *fliC* (de Vries *et al.*, 1998). However, this gene has been inserted into clone 19 in the minus orientation and therefore flagellin is not produced. Interestingly, the minus orientation of this insert forms an uninterrupted ORF that is fused in the correct frame at the 3' (but not 5') end. E-tag expression was negative, and this is possibly due to the incorrect fusion to the signal peptide. The gene products of *edd* and *accB* encode antigens in *H. pylori* and *Francisella tularensis*, respectively (Lazowska *et al.*, 2000; Havlasova *et al.*, 2002). The *shdA* gene (clone 140) encodes a fibronectin-binding protein that is induced *in vivo* and is essential for colonisation of PP (Kingsley *et al.*, 2002), but since the biopanning serum was obtained from mice that were inoculated i.p. rather than orally, it is unlikely that antibodies were produced to this protein.

c. Dot blots

Since no known *Salmonella* antigens were expressed by the clones in Table 4.9, a positive control was constructed in pG8SAET for the purpose of screening for new antigens from phage lysates of individual clones. M. Mogensen identified *rplL*, which encodes the L7/L12 ribosomal subunit protein, as an antigen recognised by anti-*S.* Typhimurium serum from the final bleed of BALB/c mice having been

immunised and subsequently challenged three times with live bacteria (see Chapter 3 for details). The antigenicity of L7/L12 was verified by Western blot of the purified recombinant His-tagged protein, using the same BALB/c serum (M. Mogensen, 2004, unpublished data). The L7/L12 proteins of *Brucella abortus*, *Brucella melitensis*, *Mycobacterium bovis*, and *H. pylori* L7/L12 are strongly antigenic (Bachrach *et al.*, 1994a; Bachrach *et al.*, 1994b; Kitaura *et al.*, 1999; Kimmel *et al.*, 2000). This antigenicity is not unexpected given the fact that ribosomes are highly expressed, form large molecular complexes, and constitute target antigens. Indeed, ribosomal vaccines have been prepared from over 28 different species of bacteria, conferring a high degree of protection, although the basis of the protective response has not been established clearly (Gregory, 1986). L7/L12 was therefore an attractive positive control for screening phage lysates. Its small size was ideal since the average insert size of the phagemid clones to be screened was also small.

Phage lysates were made of each clone with an insert having a plus orientation, in addition to clones for which no sequence data was available. These clones, screened as dot blots, are identified in Table 4.9 with bold type. Repeated screening with independently produced lysates ensured that any candidate antigens would give reproducible positive results prior to further analysis. Figure 4.5 (D), which shows the relative E-tag expression levels of each clone in phage lysates of similar titre, can be used to estimate the relative expression levels of the displayed polypeptide to which the tag is fused. Thus, the different expression levels of polypeptide in dot blots are likely to affect the sensitivity of the dot blot screening procedure. However, there is no feasible way to control for varying expression levels of individual clones. Additional blocking steps designed to lower any non-specific binding were used in order to enhance discrimination between the positive and

negative controls. Additional blocking with foetal bovine serum/ bovine serum in milk solution was compared to milk alone. To avoid the possible loss of unknown antigens, foetal bovine serum was favoured over bovine serum for inclusion in the second blocking step. This procedure was adopted for screening all dot blots. Figure 4.4 shows the intensity and level of consistency with which the 10 chosen clones were positive, compared to the positive control. For this reason, these 10 were sub-cloned for verification of antigenicity (see Chapter 5).

The *cysA* gene encodes a sulphate/thiosulphate import ATP-binding protein. This protein is part of the sole sulphate transporter complex of the macrophage-tropic intracellular pathogen, *Mycobacterium tuberculosis*, and is needed for in vivo acquisition of sulphur (Wooff *et al.*, 2002). The prospect of this protein's antigenicity is tantalising since it may be induced during *Salmonella* pathogenesis. *STM3167* encodes a putative diadenosine tetraphosphate (Ap4A) hydrolase. This type of enzyme catalyzes the conversion of diadenosine tetraphosphate to adenosine diphosphate (ADP). While the function of this protein has not been investigated, another Ap4A hydrolase, ApaH, is involved in the regulation of dinucleoside polyphosphate pools. This regulation, which is also controlled by a second hydrolase YgdP, affects the ability of *S.* Typhimurium to invade cells in vitro (Ismail *et al.*, 2003).

The *ytfE* gene encodes a putative cell morphogenesis protein, which may or may be a part of the normal cell cycle since its function has not been elucidated (McClelland *et al.*, 2001). The protein encoded by *ptsN* is a phosphotransferase system enzyme IIA that plays a role in linking carbon and nitrogen assimilation (Tchieu *et al.*, 2001). The *oat* gene, also called *argD*, encodes a putative acetylornithine/succinyldiaminopimelate aminotransferase that is involved in the

fourth step of the arginine and lysine biosynthetic pathways (McClelland *et al.*, 2001). The P22 bacteriophage gene *gp16* encodes a protein that is required for injection of the phage genome into the host cell (Umlauf and Dreiseikelmann, 1992). The *yijO* gene encodes a hypothetical transcriptional regulator of the AraC/XylS family. This family of proteins is characterised by having a helix-turn-helix motif, typically activating transcriptional activity in response to environmental signals (Gallegos *et al.*, 1997). *STM2235* encodes an uncharacterised putative phage protein, while *yhjJ* encodes a putative periplasmic peptidase (McClelland *et al.*, 2001). The protein encoded by *ydiY* is a putative outer membrane protein that is induced by salt/ low pH, making it a possible candidate for *in vivo* induction of expression (McClelland *et al.*, 2001; Stancik *et al.*, 2002). Sub-cloning and subsequent purification of these ten fragments containing possible epitopes will allow immunological tests to determine whether they are truly antigens.

CHAPTER 5

Cloning, Purification, and Immunodetection of Candidate Antigens

A. Introduction

In Chapter 4, ten phagemid clones displaying putative antigens were identified after biopanning the pG8SAET library and screening individual clones with hyperimmune anti-*S.* Typhimurium serum. This chapter focuses on verifying whether the protein fragments displayed by these clones are truly antigens. In order to do this, the corresponding sequences need to be sub-cloned, expressed, purified, and tested by immunoblot and ELISA. The experiments performed and results obtained are discussed below.

1. TOPO and Gateway Cloning

In this chapter, TOPO® and Gateway® cloning are used to construct expression vectors encoding his-tagged protein fragments for subsequent purification on nickel resin. This cloning is achieved using the vectors pENTR/SD/D-TOPO and pET-DEST 42, respectively, supplied by Invitrogen. Selection markers for these two vectors are kanamycin resistance (Kan^R) and ampicillin resistance (Amp^R), respectively. Unmodified pET-DEST 42 also contains chloramphenicol resistance (Cm^R) for counter-selection.

TOPO® cloning exploits the properties of topoisomerase I, which catalyzes the cleavage of the DNA phosphodiester backbone and covalently attaches to the 3' phosphate. The vector pENTR/SD/D-TOPO is supplied as a linearised plasmid with topoisomerase I covalently attached at each end. Topoisomerase I is released when the 5' hydroxyl group of a DNA strand attacks the phospho-tyrosyl bond to re-form the DNA backbone. The energy conserved by the formation of the double bond

between the DNA and enzyme thus confers a kinetic advantage that can be used to design rapid ligation reactions. Directional cloning is made possible by a 4-base single-stranded 3' overhang that is designed to invade the end of a double-stranded DNA molecule containing the complementary sequence. In this vector, the overhang is positioned to invade the beginning of the sequence to be cloned (Figure 5.1) (Invitrogen, 2003b).

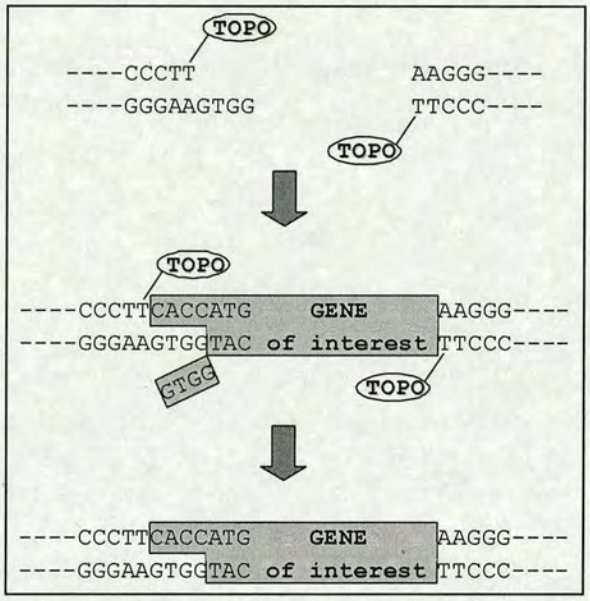


Figure 5.1. Schematic representation of directional TOPO® cloning. A 3' overhang in the linearised vector invades the end of a double-stranded DNA molecule containing the complementary CACC sequence, and the displaced GTGG is then cleaved off in *E. coli* host cells.

Gateway® cloning utilises the site-specific integration and excision reactions that are part of the bacteriophage λ lysogenic and lytic pathways. Recombination between site-specific attachment sites *att*P (from λ phage) and *E. coli att*B gives rise to the hybrid sites *att*L and *att*R. This reaction is catalysed by λ integrase and the *E. coli* integration host factor (IHF) protein and is part of the lysogenic pathway. The reverse of this reaction is catalysed by λ integrase, λ excisionase, and *E. coli* IHF to produce *att*P and *att*B (Invitrogen, 2003a).

The cloning site of pENTR/SD/D-TOPO is flanked by a pair of recombination sites, *att*L1 and *att*L2. The vector pET-DEST 42 contains *ccdB* and Cm^R flanked by *att*R1 and *att*R2, followed by a sequence encoding a V5 epitope and 6× histidine tag. The modifications that produced *att*L1 and *att*L2 confer specificity for *att*R1 and *att*R2, respectively, resulting in directional recombination. The *ccdB* gene encodes a protein that interferes with *E. coli* DNA gyrase and therefore inhibits the growth of the cell that expresses it. Recombination between pENTR/SD/D-TOPO and pET-DEST 42 results in an exchange of *ccdB* and Cm^R for the gene having been cloned into pENTR/SD/D-TOPO (Figure 5.2). Transformation of this reaction into most *E. coli* strains (which are unable to grow if they express *ccdB* from unmodified pET-DEST 42) and subsequent propagation on ampicillin-containing medium (eliminating the entry vector) favours the growth of the expression clone (Invitrogen, 2003a).

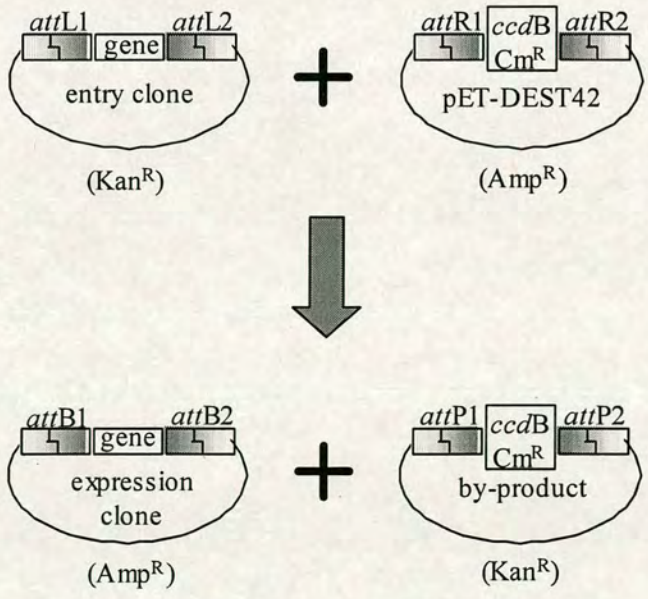


Figure 5.2. Generation of an expression clone from an entry vector using the Gateway® recombination reaction. The product of the *ccdB* gene (present in parental pET-DEST42 and the reaction by-product) are toxic when transformed into certain *E. coli* strains, such as DH5α.

2. Purification

Protein purification was traditionally based on the sequential partitioning of a protein mixture on the basis of size, hydrophobicity, isoelectric point and/or affinity. Recombinant DNA technology allowed for the manipulation of sequences to produce proteins fused to peptides and polypeptides that function as tags for one-step affinity purification. These tags include maltose-binding protein, glutathione *S*-transferase, and the polyhistidine tag. The affinity tag makes the recombinant protein unique in a bacterial lysate and allows binding to its target ligand with a high degree of specificity. Selection of the affinity tag depends on the nature of the intended purification and the application to follow. The 6× polyhistidine tag is small, soluble, and uncharged at physiological pH, making it less likely to interfere with the structure of the protein to which it is fused. Additionally, this tag can be used under native as well as denaturing conditions since its interaction with the target ligand is not conformation-dependent. The ligand used for purification of polyhistidine-tagged proteins is the nickel ion. The purifications described in this chapter use Ni-NTA agarose beads (Qiagen). Nitrilotriacetic acid (NTA) is a tetradentate chelating agent that occupies four of the six available binding sites of nickel. A nitrogen atom from each of two adjacent histidines of the tag interacts with the two free sites of the nickel ion, thus tethering the fusion protein to the Ni-NTA agarose beads. The interaction between fusion protein and Ni-NTA agarose beads can be disrupted with a high concentration of imidazole or low pH, thus eluting the protein (Qiagen, 2003).

Although affinity tags greatly reduce the number of purification steps needed, an additional purification step may be necessary to ensure the purity of the protein in some cases. In this chapter, an additional purification step involving ion exchange

chromatography (IEC) is used. IEC separates proteins according to isoelectric point (pI) and is dependent on the pH and salt concentration of the fluid phase. The overall charge on a protein depends on the pH of the buffer in which it is dissolved. Adjusting the pH of the protein solution can favour binding of the target protein to the charged groups of the column, while simultaneously limiting the binding of other proteins. Subsequent application of a buffer with increasing salt concentration provides ions that compete with the target protein for binding to the column until it is eluted. As the ionic strength increases, each bound protein elutes at a specific salt concentration until all proteins are eluted (Amersham, 2004). Mono Q beads (Amersham Biosciences) are positively charged and thus exchange anions; Mono S beads (Amersham Biosciences) are negatively charged and thus exchange cations.

B. Results

1. Cloning and expression of His-tagged protein fragments

The insert sequences of the ten phagemid clones isolated after biopanning and screening were the basis for sub-cloning the corresponding gene fragments. All forward primers began with CACC at the 5' terminus for directional cloning of the PCR product into the pENTR/SD/D-TOPO vector, followed by an ATG for translation initiation of the gene fragment. Some sub-cloned fragments included extra coding sequence from the corresponding genes due to minor adjustments during primer design for improved DNA amplification. The amino acid sequence of each gene, the portions identified in phagemid clones, and the portions chosen for sub-cloning are indicated in Table 5.1.

Using the primers listed in Table 2.4 (see Chapter 2), the ten gene fragments were amplified with Pfu polymerase (Promega) from *S.* Typhimurium SL1344 DNA and cloned into pENTR/SD/D-TOPO. Subsequently, purified plasmid DNA of all ten clones was digested with *Bsr*GI (which cleaves DNA at *att*L1, *att*L2, *att*B1, *att*B2) and analysed by agarose gel electrophoresis (Figure 5.3A). After confirming that the entry vectors contained inserts of the correct size, the cloned sequences were transferred to pET-DEST 42 by recombination as described above for Gateway cloning. After *Bsr*GI digestion of the resulting expression clones (Figure 5.3B), each was sequenced with "oligo1" to confirm that the cloned DNA was correctly fused to the vector at both ends and contained no mutations.

1. AAL21335 CysA (clone 9)

MSIEIARIKK SFGRTQVLND ISLDIPSGQM VALLGPSGSG KTTLLRIIAG LEHQSSGHIR FHGTDVSRLH
ARERKVGFVF QHYALFRHMT VFDNIAFGLT VLPRRDRPTA AAIKTKVTQL LEMVQLAHLA DRFPAQLSGG
QKQRVALARA LAVEPQILLL DEPFGALDAQ VRKELRRWLR QLHEELKFTS VFVTHDQEEA TEVADRVVVM
SQGNIEQADA PDRVWREPAT RFVLEFMGEV NRLTGTVRGG QFHVGAHRWP LGYTPAYQGP VDLFLRPWEV
DISRRTSLDS PLPVQVIEAS PKGHYTQLVV QPLGWYHDPL TVVMAGEDVP VRGERLFVGL QKARLYNGDQ
RIETREEELA LAQSA

2. AAL22041 STM3167 (clone 25)

MTCIFCQIVE GKAPCHKVWE DEHHLAFLSI FPNTDGFTVV IPKKHYPSYA FDLPPQALAD LMLATQKVAK
KLDKAFPDVS RTGMFFEGFG VDHVHSKLSP MHGTGDLTHW KPIESRQNKF FEQYEGYLSS HDHERADDEK
LAALAARIRE A

3. AAL23219 YtfE (clone 53)

MAYRDQPLGE LALSIPRASA LFRQYDMDYC CGGKQTLARA AARHDVDIDI IEAQLAQLAE QPIEKDWRAV
PLADIIDHIV VRYHDRHREQ LPELILQATK VERVHADKPN VPRGLTKYLT ALHEELSSHM MKEEQILFPM
IKQGMGRQAT GPISVMESEH DEAGELVDVI KHVTKNVTPP PEACTTWKAM YNGINEMIDD LMEHISLENN
VLFPRALAGE

4. AAL22191 PtsN (clone 113)

MINNDTTLQL SSVLNQECTR SGVHCQSKKR ALEIISELAA KQLSLPPQVV FEAILTREKM GSTGIGNGIA
IPHGKLEEDT LRAVGVFVQL ETPIAFDAID NQPVDLLFAL LVPADQTKTH LHTLSLVAKR LADKTICRRL
RAALNDEELY QIITDTEGEQ NEA

5. AAL22091 Oat (clone 114)

MKALNREVID YFKEHVNPGF LEYRKSVTAG GDYGAVEWQA GSLNTLVDTQ GQEFIDCLGG FGIFNVGHRN
PVVVSAVQNQ LAKQPLHSQE LLDPLRAMLA KTLAALTPGK LKYSFFCNSG TESVEAALKL AKAYQSPRGK
FTFIATSGAF HGKSLGALSA TAKSTFRRPF MPLLPGFRHV PFGNIDAMSM AFSEGKKTGD EIAAVILEPI
QGEGGVILPP QGYLTEVRKL CDEFGALMIL DEVQTGMGRT GKMFACEHEN VQPDILCLAK ALGGGVMPIG
ATIATEEVFS VLFDNPFLHT TTFGGNPLAC AAALATINVL LEQNLPAQAE QKGDTLLDGF RQLAREYPNL
VHDARGKGML MAIEFVDNET GYRFASEMFR QRVLVAGTLN NAKTIRIEPP LTLTIELCEQ VLKSARNALA
AMQVSVEEV

6. AAN87103 Gp16 of P22 phage (clone 122)

MKVTANGKTF NFPDGTSTED IGAAVDEYFA GQASAAETQP AEQQEEPQQP EQSLMQRAGD LLTGGQSAGQ
IAEQAGRGLV NIPFDVLQGG ASLINAISQG LGGPKVLDDV YRPVDRPTDP YAQAGESIGG YLIPGAGVAG
NMAIGSVAEA ANQQGDFAGN VAKNAAVNLG AQGLLSGAAK LVGRGITAAR GEIAPEARQL IDTAESMGVK
PMTSDMIKPG NAFTRSLMQG GEGALLGTGG KRAEQYAIRS KLLGDYFDRV GGYNPDDIVK SMTSTVGGRK
NAAGAVRDEI VNRMGSAPVG TTNSINAIDT NIARLEKLGT SADQRLLTAL KNLKGELNSG NVDFDLLQQH
RTAFRTNVQG DAMVFPNQAK AATNMVENAM TRDLRNAVGK SLGPQDAAKY LKSNSDFANI YNKVLNKRIS
NTLNKARSEY TPELINTVVF SRKPSDIKRI WSSLDNKGKD AMRAAYISKI AEKTGDSPAK FITEVNKLKA
QSGGEIYNTI FSGRHMKELD ALHDVLRQTA RSDSANVVTQ TGQALANPVR LGAAIPTLGK SLAAEAGYGL
AMRVYESKPI RNMLLRLANT KPGTPAYERA LNQAATAVRP LLANEATRQ

Table 5.1. Amino acid sequence of genes chosen for sub-cloning. The phagemid clones associated with the encoded proteins are indicated in parentheses. Amino acids encoded by the phagemid clones are underlined, and the portions to be expressed by sub-clones are in bold type. The sequence of clone 122 contains two point mutations relative to the published P22 sequence that produce A→D and S→A substitutions. These amino acids are doubly underlined. Constructs in pET-DEST 42 are predicted to have KGGRADPAFLYKVVINSKLEGKPIPNPLLGLDSTRTGHHHHHH attached to the C-terminus of protein fragments.

7. AAL 22956 YijO (clone 126)

MYHDVSHLLS RLINGPLPLR QIYFASASGP APELAYQVDF PRLEIVLEGE LTDMSITAPL IPCDVLYVPA
GGWNIPQWQT PVTTLSILFG KQQLGFSVVH WDGQQHQNLT KQHVARRGPR IGSFLLQTLN EMQMQPQEQQ
TARLIVASLL SHCRDLLGSQ IQTASRSRAL FEAIREYIDE
RYAAPLTRES VAQAFYISPN YLSHLFQKTG AIGFNEYLNH TRLEHAKTLL KGYDLKVKEV AHRCGFVDSN
YFCRLFRKNT ERSPSEYRRQ YHSQLTEKQI TPG

8. AAL21137 STM2235 (clone 148)

MHRIDTPTAQ KDKFGQGKNG FTNGDPATGR RATDLNSDMW DAVQEEVCTV IEAAGIPLSK GEHTQLHAAI
GRLIYEQVKT RLEKNQNGAD IPNKPLFLQN VGLVDVLFKG DGRFLAGTFV SDAIDRTSIG ARAATGCQFM
RAHQAPDAPD QVSFWQIITL SEVVSPTTVV DVLAVSGNNV LFGHGTGTGI TSWRQVAMLE GGAFTGGISA
PNMRGDTLVT VGDGTGGMAK GDVDGAGFNG
NNLNIKSWNG IGFQNSEDLA IRAYISTRLG VIAAAENLQA GSAIFNKNGD VYGDIWGGGS GPGWLSAFVA
SKPARQYITM VGVYQNDKTK PFMLHDDGSG VFLATTDMLS GYVQSIRFGA VEHGNLYRSP GFADQLGYVI
TGVENGDSND TPDRIQRRLL QLKVNGQWYT VGA

9. AAL20252 YdiY (clone 134)

MKLLKAVPAV VMLAGGVFAS LYAAADDSVF TVMDDPSTAK KPFEGNLNAG YLAQSGNTKS SSLTADTAMT
WYGQRTAWSL WGNASNTSSN DERSSEKYAV GARNRFNMTD YDYTFGQASW LTDRFNGYRQ RDVLTAGYGR
QFLNGPVHSF RFEFGPGVRY DEHTDDTTET QPLGYASGSY AWQLTDNAKF TQGVSVFGAE DTTLNSETAL
NVAINEHFGL KVGYNLTWNS QPPESAPEHT DRRTTVTLGY KM

10. AAL22473 YhjJ (clone 118)

MQGTKIRLLA GSLLMLASAG YVQADALQPD PAWQQGTLAN GLQWQVLATP QRPSDRIEVR LQVNTGSLTE
STQQSGFSHA IPRIALTQSG GLDAAQARSL WQQGFDPKRP MPPVIVSYDS TLYNLSLPNN RNDLLKEALT
YLANVSGKLT ITPETVNHAL SSEDMVATWP ADTKEGWWRY RLKGSALLGH DPAEPLKQPV DAAKIQAFYE
KWYTPDAMTL IVVGNIDARS VAEQINKTFG TLKGKRETPA PVPTLSPLRA ESVSIMTDAV RQDRLSIMWD
TPWQPIRESA ALLRYWQADL AREALFWHIQ QELTKNNAKD IGLGFDCRVL FLRAQCAINI ESPNDKLNTN
LSLVANELAK VRDKGLSEEE FTALVAQKNL ELQKLFATYA RTDTDILTGQ RMRSLQNQVV DIAPEQYQKL
RQNFLNSLTV DMLNQNLRQQ LSQEMALILL QPQGEPEFNM KALKATWDEI MVPTTAAAVE ADEAHPEVTE
TPAAQ

Table 5.1 (continued). Amino acid sequence of genes chosen for sub-cloning. The phagemid clones associated with the encoded proteins are indicated in parentheses. Amino acids encoded by the phagemid clones are underlined, and the portions to be expressed by sub-clones are in bold type. Constructs in pET-DEST 42 are predicted to have **KGGRADPAFLYKVVINSKLEGKPIPNPLLGLDSTRTGHHHHHH** attached to the C-terminus of protein fragments.

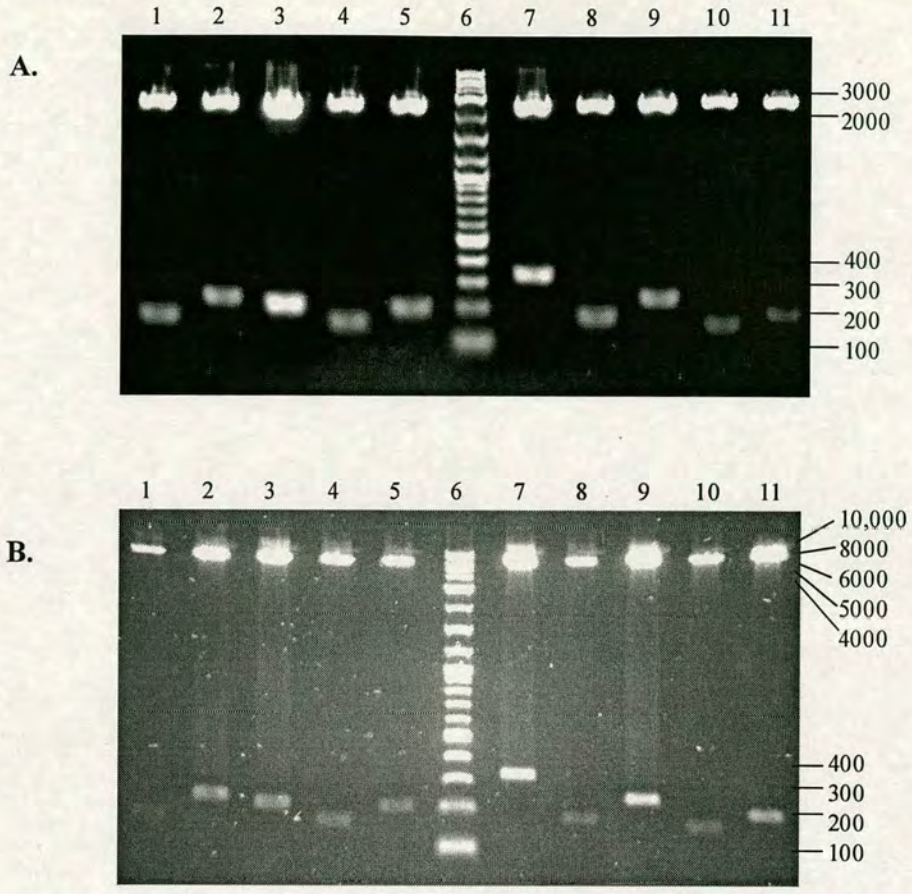


Figure 5.3. Restriction analysis of (A) entry and (B) destination clones. Digestion of plasmid DNA with *Bsr*GI released fragments from entry (~2600 bp) and destination (~7400 bp) vectors. These fragments correspond in size to the cloned DNA plus approximately 80 nucleotides from the vector. *Bsr*GI-released fragments are identical for corresponding entry and destination clones because the restriction sites are located within the *att* sites that flank the cloned DNA. Sizes (in bp) represented by bands of the molecular weight marker (lane 6) are indicated on the right. Lanes 1-5 are digested plasmids containing sequences from *cysA*, *STM3167*, *ytfE*, *ptsN*, and *oat*, respectively. Lanes 7-11 are digested plasmids containing sequences from *gp16*, *yijO*, *STM2235*, *ydiY*, and *yhjJ*, respectively.

Sequencing of all pET-DEST 42 expression clones verified that the inserted sequence of each was correct. The two point mutations in *gp16* that identified the difference between the phagemid clone 122 insert and the published P22 bacteriophage sequence for this gene, were also present in the independently produced expression clone. After confirmation of sequence accuracy, optimal

conditions for induction of protein expression were experimentally assessed for all ten clones in *E. coli* BL21 (DE3) cells. The vectors pVCN2 and pMOG3 (see Table 2.3 of Chapter 2), encoding 6× His-tagged green fluorescent protein (GFP) and 6× His-tagged L7/L12 ribosomal subunit protein, respectively, were used as negative and positive controls for expression, purification, and testing of potential antigens. Test inductions were performed in 10 ml cultures with 1 mM IPTG for 2-18 hrs at 18-37°C, and sonicated samples were analysed by SDS-PAGE. Moderate induction was observed for the positive control. Low-level induction was achieved for the negative control, as well as for constructs of *cysA*, *gp16*, *STM2235*, and *yhjJ* (Figure 5.4). Induction in the six other constructs could not be detected by SDS-PAGE for any length of time at any temperature tested (data not shown). A western blot of cell lysates with anti-His antibody could not detect the affinity tag in lysates from these six constructs, while the induced *STM2235* construct was strongly apparent (Figure 5.5).

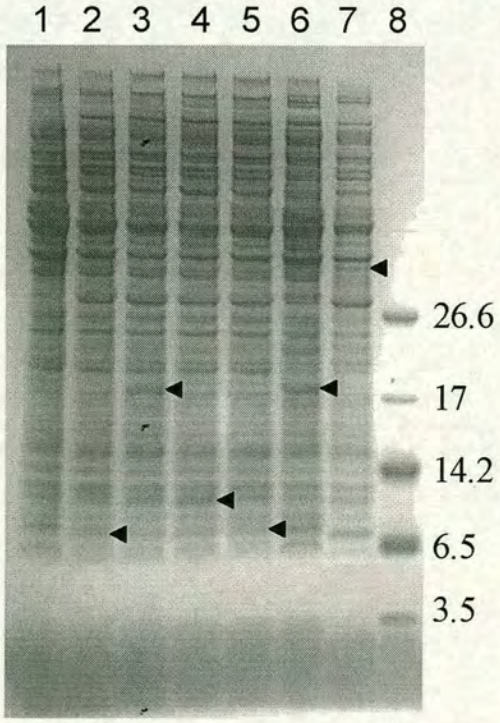


Figure 5.4. Sonicated *E. coli* with IPTG-induced expression of His-tagged proteins and protein fragments. Lane 1: uninduced; Lane 2: CysA frag at 4 hrs, Lane 3: Gp16 at 2 hrs; Lane 4: STM2235 at 2 hrs; Lane 5: YhjJ frag at 2hrs; Lane 6: L7/L12 at 20 hrs; Lane 7: GFP at 18 hrs; Lane 8: Molecular weight marker, with band sizes (in kDa) indicated on the right. Induced bands are indicated with arrows. GFP and L7/L12 were induced at 18°C; all others were induced at 37°C.

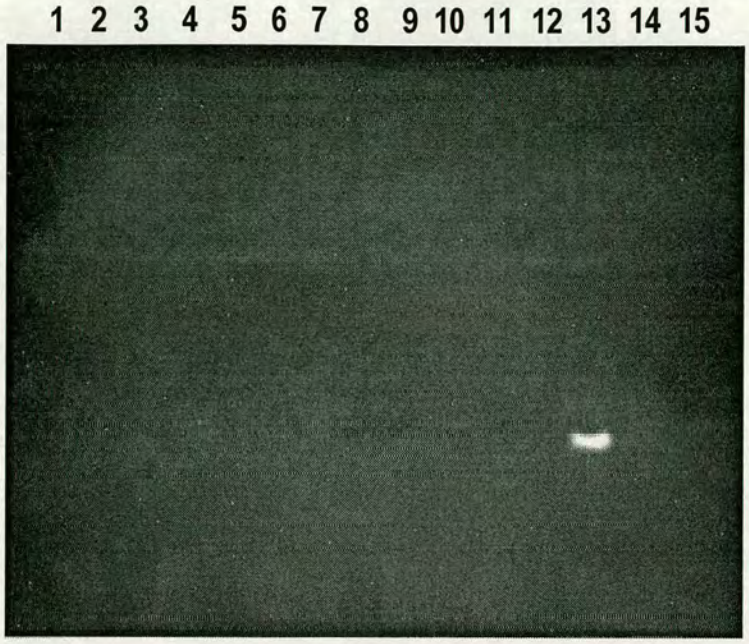


Figure 5.5. ECL of Western blot of IPTG-treated, sonicated *E. coli* carrying constructs encoding His-tagged protein fragments. Lane 1: uninduced; Lanes 2, 3: STM3167 frag at 2 hrs, 4 hrs; Lanes 4, 5: YtfE frag at 2 hrs, 4 hrs; Lanes 6, 7: PtsN frag at 2 hrs, 4 hrs; Lane 8: molecular weight marker (drawn in after staining with Ponceau S and not visible during ECL detection procedure), Lanes 9, 10: Oat frag at 2 hrs, 4 hrs; Lanes 11, 12: YijO frag at 2 hrs, 4 hrs; Lane 13: STM2235 frag at 2 hrs, Lanes 14, 15: YdiY frag at 2 hrs, 4 hrs. All inductions for this figure were performed at 37°C with 1 mM IPTG.

2. Purification

a. Affinity purification on Ni-NTA agarose in native conditions

After assessment of results from small-scale inductions, expression of His-tagged proteins and protein fragments was induced in 500 mL cultures. These cultures were induced with 1 mM IPTG for 2 hrs at 37°C (Gp16 frag, STM2235 frag, and YhjJ frag), 4 hrs at 37°C (CysA frag), or 18 hrs at 18°C (STM3167 frag, YtfE frag, PtsN frag, Oat frag, YijO frag, YdiY frag, L7/L12, and GFP). Cells from all cultures were then pelleted, resuspended in Lysis Buffer, and lysed with a French

press. The lysates were combined with Ni-NTA agarose beads and mixed for 3 hrs at 4°C to allow binding of the His-tagged fusions, after which the beads were washed several times to remove unbound protein. The beads from CysA frag, Gp16 frag, STM2235 frag, and YhjJ frag lysates were then washed several times and subsequently eluted sequentially with buffer containing 0.05 M, 0.1 M, 0.15 M, and 0.2 M imidazole. Sequential elutions were analysed by SDS-PAGE to assess relative purity (Figure 5.6). All other fusions were eluted with 0.2 M imidazole. No bands corresponding to the expected molecular weight for STM3167 frag, YtfE frag, PtsN frag, Oat frag, YijO frag, or YdiY frag were apparent in the elutions from these lysates (data not shown). Elutions of L7/L12 and GFP were of a much higher degree of purity than all other elutions (Figure 5.7). The sequential elutions with different imidazole concentrations were combined for each of the four successfully induced protein fragment constructs. These, as well as the positive and negative controls, were then prepared for further purification by ion exchange chromatography. Table 5.2 summarizes calculated data and results for the ten constructs and two controls.

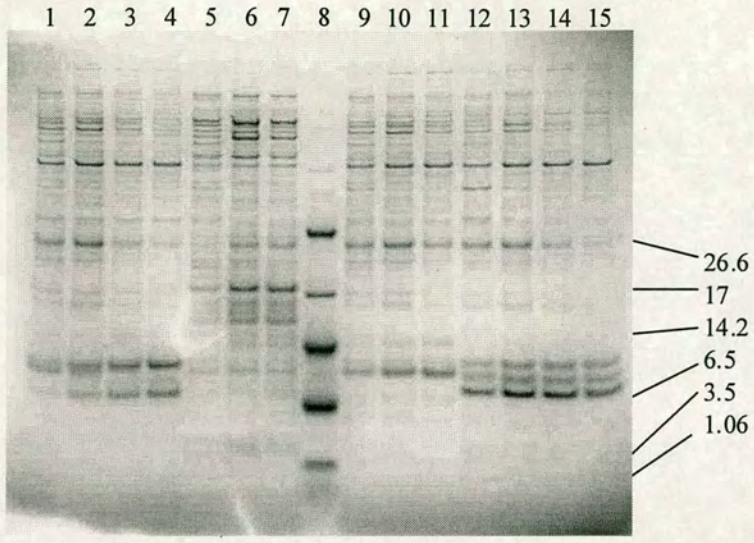


Figure 5.6. Elution of His-tagged protein fragments from Ni-NTA agarose with different concentrations of imidazole. Lanes 1-4: CysA frag eluted with 0.05 M, 0.1 M, 0.15 M, and 0.2 M imidazole; Lanes 5-7: Gp16 frag eluted with 0.1 M, 0.15 M, and 0.2 M imidazole; Lane 8: Molecular weight marker, with corresponding band sizes (in kDa) on the right; Lanes 9-11: STM2235 frag eluted with 0.1 M, 0.15 M, and 0.2 M imidazole; Lanes 12-15: YdiY frag eluted with 0.05 M, 0.1 M, 0.15 M, and 0.2 M imidazole.

Figure 5.7. Elutions of His-tagged L7/L12 and GFP from Ni-NTA with 0.2 M imidazole. L7/L12 (left) and GFP (right) are indicated with arrows.

Vector name	Ref. pG8SAET clone	Length (aa)	Calc. MW (Da)	Detectable induction?	Ni-NTA Partial Purification?	Calc. pI	Calc. pI (+His)	Calc. pI (+Etag)
pET-DEST42 cysA frag	9	76	8,444	Y	Y	5.13	8.33	4.91
pET-DEST42 STM3167 frag	25	97	10,988	N	N	5.76	8.29	5.39
pET-DEST42 ytfE frag	53	91	10,322	N	N	6.18	9.57	5.66
pET-DEST42 ptsN frag	113	75	8,306	N	N	5.57	7.28	5.09
pET-DEST42 oat frag	114	88	9,629	N	N	8.40	10.00	6.31
pET-DEST42 gp16 frag	122	129	14,404	Y	Y	9.85	10.14	9.25
pET-DEST42 yijO frag	126	80	8,993	N	N	5.77	9.40	5.19
pET-DEST42 STM2235 frag	148	100	10,752	Y	Y	5.33	9.30	4.96
pET-DEST42 ydiY frag	134	73	7,940	N	N	5.99	9.78	5.13
pET-DEST42 yhjJ frag	118	83	9,316	Y	Y	5.08	9.06	4.92
pMOG3 (see methods)	positive control	164	17,027	Y	Y	4.55	5.70	4.60
pVCN2 (see methods)	--	278	31,450	Y	Y	N/A	6.24	N/A

Table 5.2. Summary of fusion constructs, relevant properties, and expression/purification results. The predicted isoelectric point (Calc. pI) of the relevant product is represented as it would be without extra sequence, with added pET-DEST42 sequence including polyhistidine tag (+His), and as an E-tag and phage protein GP8 fusion (+Etag).

b. Purification of positive and negative controls using ion exchange

To further purify the fusion protein fragments and controls, the buffers of Ni-NTA eluates for each fusion were exchanged for the buffers to be used in IEC. L7/L12 and GFP were dialysed in 20 mM Tris-Cl (pH 8.0) in preparation for anion exchange on a Mono Q column (Amersham). Following this, the two proteins were purified consecutively on the same column. Briefly, the sample was loaded in 2 × 5 ml volumes, after which the column was washed with 10 ml of the same Tris buffer.

Following this, a gradient of the same Tris buffer with increasing ionic strength was applied over 20 ml (L7/L12) or 30 ml (GFP) until the maximum NaCl concentration of 1 M was reached. A further 10 ml of buffered 1 M NaCl was passed through the column to elute any remaining bound protein. Consecutive fractions of 1 ml were collected starting just before and ending just after the gradient was applied. Green fractions were collected from the GFP purification and analysed by SDS-PAGE (Figure 5.8). The UV absorbance value of L7/L12 elution fractions was monitored, and fractions with relatively high absorbance values were subsequently analysed by SDS-PAGE (Figure 5.9). Lanes containing fractions that were subsequently used for immunodetection are indicated in bold type.

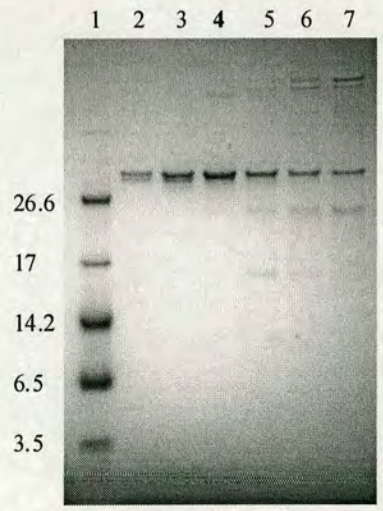


Figure 5.8. SDS-PAGE analysis of IEC eluted fractions of His-tagged GFP. Fractions (1 ml) were collected during application of a salt gradient to a Mono Q column with bound GFP fusion protein. Lanes 2-7: samples of green fractions; Lane 1: Molecular weight marker, with corresponding sizes (in kDa) on the left. The fraction in Lane 4 was subsequently used in immunodetection.

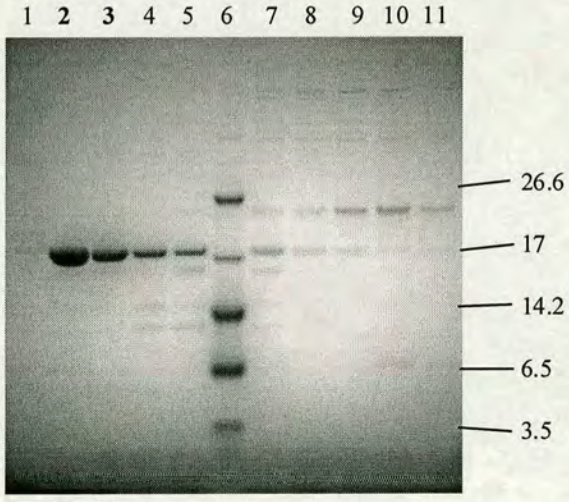


Figure 5.9. SDS-PAGE analysis of IEC eluted fractions of His-tagged L7/L12. Fractions (1 ml) were collected during application of a salt gradient to a Mono Q column with bound L7/L12 fusion protein. Lanes 1-5, 7-11: samples of consecutive fractions that exhibited the highest UV absorbance readings. Lane 6: Molecular weight marker,with corresponding sizes (in kDa) on the right. Fractions in lanes 2 and 3 were combined and subsequently used in immunodetection.

c. Purification of YhjJ frag and CysA frag fusions using ion exchange

Buffers of Ni-NTA eluates containing YhjJ frag and CysA frag were transferred to 50 mM sodium phosphate (pH 7.0) for cation exchange on a Mono S column (Amersham). Due to their low molecular weights, the buffer exchange was performed using 1 kDa cutoff spin columns. Otherwise, the IEC purification procedure for these two fusions were as described for the control fusion proteins in the previous section. UV absorbance of eluate was observed, and fractions corresponding to an absorbance peak (Figure 5.10) were analysed by SDS-PAGE (Figures 5.11). Lane 4 of Figure 5.11 contains the fraction of YhjJ frag that was sufficiently pure for subsequent use in immunodetection.

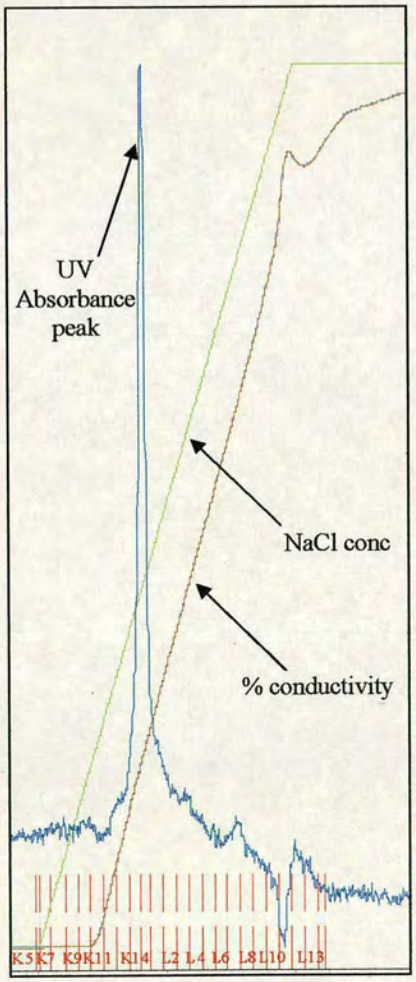


Figure 5.10. UV absorbance peak of fractions collected during the application of a salt gradient to Mono S beads to elute bound YhjJ frag. Ni-NTA purified YhjJ frag was bound to Mono S beads in buffer with no salt, and subsequently eluted with increasing salt concentrations. Fractions (indicated by red lines) corresponding to the major UV absorbance peak were subsequently analysed by SDS-PAGE in Figure 5.11.

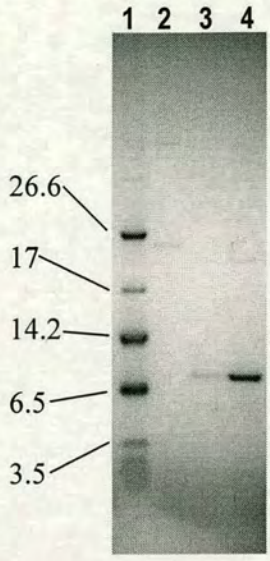


Figure 5.11. SDS-PAGE analysis of IEC eluted fractions corresponding to a major UV absorbance peak indicating the presence of protein. Elution fractions were analysed by SDS-PAGE to assess purity and molecular weight of the protein detected by the major absorbance peak in Figure 5.10. Lane 1: Molecular weight marker with corresponding band sizes (in kDa) indicated on the left. Lanes 2-4: Samples of fractions corresponding to the major UV absorbance peak measured during IEC purification of YhjJ frag. Lane 4 contains the fraction used subsequently for immunodetection experiments.

The CysA frag Ni-NTA was similarly subjected to further purification by IEC on a Mono S column. Fractions corresponding to Peak A in the CysA frag IEC elution contained two bands, the lower of which matched the expected MW for the protein fragment fusion. The Peak A fractions were combined and re-purified by IEC using the same procedure as was used for the first IEC purification of CysA frag. This second purification produced a single, barely detectable absorbance peak that was analysed by SDS-PAGE and found to consist of only the upper contaminating band from the Peak A fractions (data not shown). Therefore, a new purification was performed from a fresh 500 ml culture, under denaturing conditions. This and the following purifications under denaturing conditions were performed, due in part to limited time availability for experimental work.

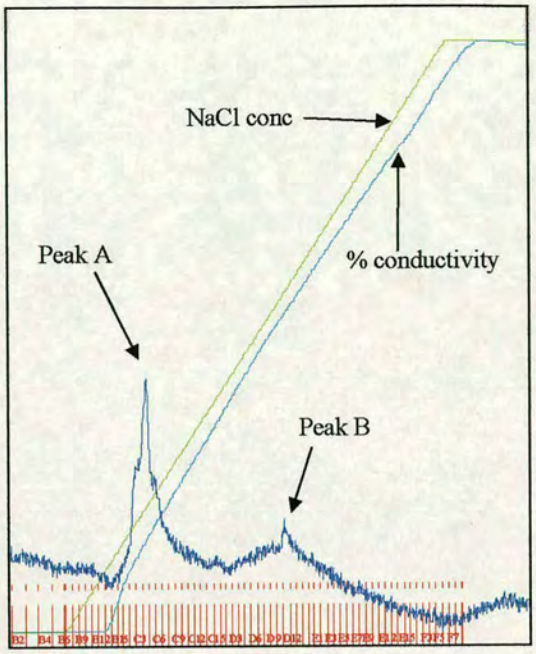


Figure 5.12. UV absorbance peaks of fractions collected during the application of a salt gradient to Mono S beads to elute bound CysA frag. Ni-NTA purified CysA frag was bound to Mono S beads in buffer with no salt, and subsequently eluted with increasing salt concentrations. Fractions (indicated by red lines) corresponding to UV absorbance peaks A and B were subsequently analysed by SDS-PAGE in Figure 5.13.

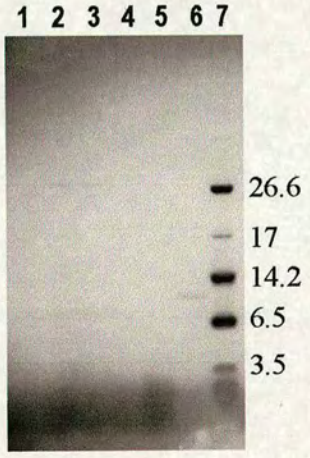


Figure 5.13. SDS-PAGE analysis of IEC eluted fractions corresponding to UV absorbance peaks indicating the presence of protein. Lanes 1-5: samples of fractions corresponding to Peak A in Figure 5.12. Lane 6: Sample of a fraction corresponding to Peak B. Lane 7: Molecular weight marker, with corresponding sizes (in kDa) on the right.

Cells were pelleted and lysed with Denaturing Lysis Buffer, washed with Denaturing Wash Buffer and then with Refolding Buffer, and eluted again in Refolding Buffer with added 0.2 M imidazole. The eluate was analysed by SDS-PAGE (Figure 5.14). To remove the contaminating protein bands that co-purified,

this elution was re-purified by IEC using the same procedure as was used for the first IEC purification. Elution fractions of 0.5 ml were collected, and SDS-PAGE was performed on samples of fractions with increased UV absorbance (Figures 5.15, 5.16). Although the concentration was quite low, a single band of the correct size was isolated. The three indicated fractions were combined and subsequently concentrated by lyophilisation followed by resuspension in water. This preparation was subsequently used for immunodetection.

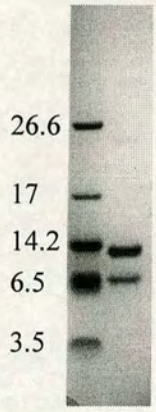


Figure 5.14. SDS-PAGE analysis of CysA frag Ni-NTA affinity purification under denaturing conditions. CysA frag was affinity purified from bacterial culture using denaturing and refolding buffers. Left lane: Molecular weight marker, with band sizes (in kDa) indicated on the left. Right lane: Elution containing the CysA frag fusion.

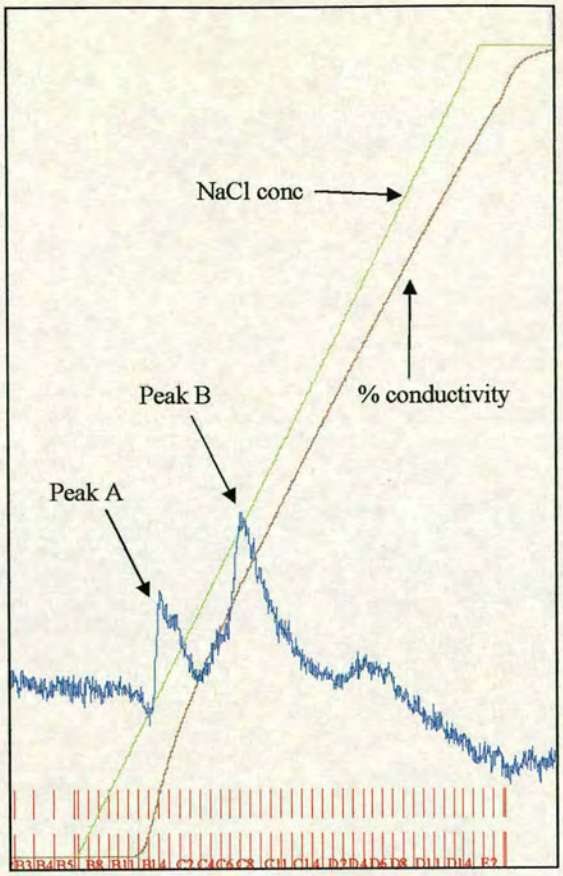


Figure 5.15. IEC separation of Mono S-bound proteins obtained by elution of His-tagged protein originally bound to Ni-NTA from bacterial lysate in denaturing conditions. Ni-NTA purified CysA frag was bound to Mono S beads in buffer with no salt, and subsequently eluted with increasing salt concentrations. Fractions (indicated by red lines) corresponding to Peak B were subsequently analysed by SDS-PAGE in Figure 5.16.

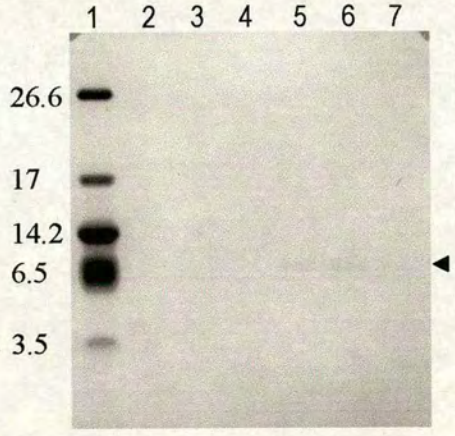


Figure 5.16. SDS-PAGE analysis of IEC elution fractions from Peak B of Figure 5.15. Lane 1: Molecular weight marker with corresponding banc sizes (in kDa) indicated on the left. Lanes 2-7: Samples from fractions associated with Peak B in Figure 5.15. The position of CysA frag is indicated with an arrow on the right. Fractions reperesented in Lanes 5-7 were combined, concentrated, and subsequently used in immunodetection.

d. Purification of Gp16 frag and STM2235 frag

Ni-NTA elutions of Gp16 frag and STM2235 frag (purified in native conditions) were dialysed in 50 mM sodium phosphate (pH 7.0) using a 3.5 kDa

cutoff membrane in preparation for cation exchange on a Mono S column. Gp16 frag and STM2235 frag precipitated during dialysis. SDS-PAGE analysis confirmed that the protein fragment fusions were contained only in the precipitate and not in the remaining soluble fraction (Figure 5.17).

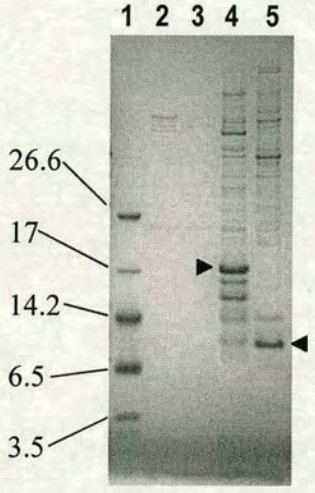


Figure 5.17. SDS-PAGE analysis of precipitates that developed during dialysis of Gp16 frag and STM2235 frag. Lane 1: Molecular weight marker, with corresponding sizes (in kDa) indicated to the left; Lanes 2, 3: remaining soluble fractions of Gp16 frag, STM2235 frag; Lanes 4, 5: precipitates of Gp16 frag, STM2235 frag. The protein fragment fusions are indicated with arrows.

Precipitates were dissolved in Denaturing Lysis Buffer containing urea, and subsequently incubated with Ni-NTA beads for a second affinity purification. Beads were washed with denaturing buffer and subsequently with refolding buffer. Elutions whith 0.2 M imidazole in refolding buffer were analysed by SDS-PAGE. No detectable protein was present in the Gp16 frag eluate; however, STM2235 frag was present, with increased purity, in its corresponding eluate (Figure 5.18).

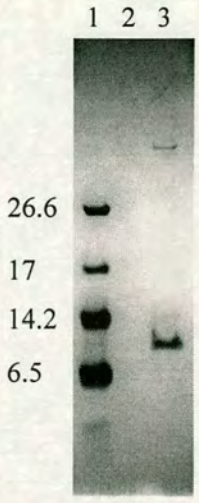


Figure 5.18. Elutions of Gp16 frag and STM2235 frag after a second affinity purification with Ni-NTA. Precipitates in Figure 5.17 were dissolved in Denaturing Lysis Buffer, and subsequently washed with Denaturing Wash Buffer followed by Refolding Buffer. Ni-NTA beads were then eluted using the same Refolding Buffer with added 0.2 M imidazole. Lane 1: Molecular weight marker with corresponding band sizes (in kDa) on the left; Lane 2: Gp16 frag elution; Lane 3: STM2235 frag elution.

Since the STM2235 frag eluate from the re-purification on Ni-NTA still contained contaminating *E. coli* proteins, the eluate was prepared once again for IEC. Instead of dialysis, the eluate was diluted tenfold in cation exchange buffer and subsequently concentrated again by polyethylene glycol (PEG)-induced extraction of the buffer through the dialysis membrane. The STM2235 frag solution was then used for IEC. STM2235 frag did not bind to the Mono S column (data not shown). Since the unbound protein solution had become very dilute, a new purification of this construct was performed from a 500 ml IPTG-induced culture. The same denaturing and refolding buffers were used in the same purification protocol. The resulting elution was analysed by SDS-PAGE (Figure 5.19).

Gp16 frag was similarly re-purified from a new culture using denaturing and re-folding conditions. Because three bands of equal intensity were present in the eluate (data not shown), this protein was prepared for further purification on the Mono S cation exchange column. The eluate was diluted in cation exchange buffer and concentrated with PEG-induced extraction through dialysis membrane in a similar manner as was done for STM2235 frag. Like STM2235, this protein did not

bind to the Mono S column (data not shown). A new round of Ni-NTA purification using denaturing and re-folding conditions was then performed from fresh culture. The resulting elution was analysed by SDS-PAGE (Figure 5.19). The same three protein bands were present in this purification as in the previous purification.

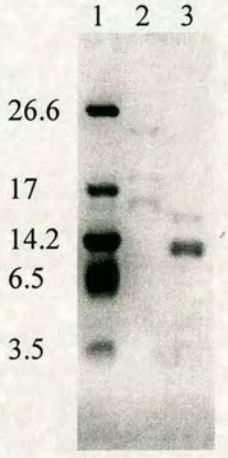


Figure 5.19. Gp16 frag and STM2235 frag affinity purified on Ni-NTA from new 500 mL cultures in denaturing followed by re-folding conditions. Elutions in Refolding Buffer with added 0.2 M imidazole are shown. Lane 1: Molecular weight marker with corresponding band sizes on the left; Lane 2: Gp16 frag; Lane 3: STM2235 frag.

Since the quantity of Gp16 frag isolated from the latest purification was very low, the same purification was repeated but with overnight binding to Ni-NTA instead of 3 hrs. The two independently obtained Gp16 frag purifications were combined into one solution and concentrated by PEG extraction of buffer through dialysis membrane. The STM2235 frag elution was used in a second, identical round of affinity purification on Ni-NTA in an attempt to remove the higher molecular weight contaminant. The resulting eluate was less pure than the original eluate (compare Gp16 frag in Figure 5.19 and Figure 5.20). Additionally, the concentrated, combined Gp16 frag purification was dominated by a protein band of approximately 25 kDa which was larger than bands seen in previous purifications (Figure 5.20). Nevertheless, this preparation was used for immunodetection.

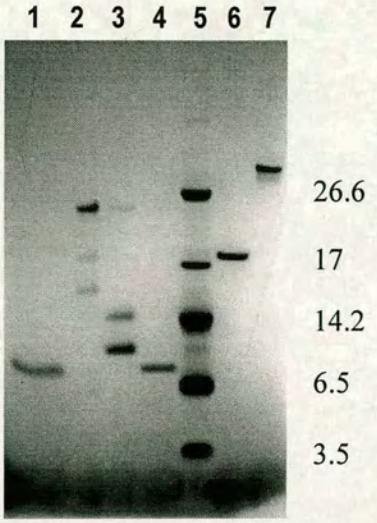


Figure 5.20. SDS-PAGE showing all purified His-tagged constructs to be used subsequently in Immunodetection. Samples (0.2 μg) of each preparation were subjected to electrophoresis, after which the gel was stained with GelCode Blue (Pierece) and imaged. Lane 1: CysA frag; Lane 2: Gp16 frag; Lane 3: STM2235 frag; Lane 4: YhjJ frag; Lane 5: Moleuclar weight marker, with corresponding band sizes (in kDa) on the right; Lane 6: L7/L12; Lane 7: GFP.

3. Immunodetection

a. ELISA

Each well of an Immulon-2 plate was coated with 0.1 μg of purified fusion protein. Pooled pre-immune and day17pc3 CBA/Ca sera were titrated against the fusion proteins, starting at a dilution of 1:40. The reaction of HRP-conjugated anti-mouse IgG with H_2O_2 and the OPD substrate caused the development of a coloured by-product for which the absorbance was measured at λ=492 nm. The absorbance values for all samples (including that of L7/L12, intended to be the positive control) were quite low; for this reason, cutoff values were not used. Absorbance values, less the average value of secondary antibody control wells, are represented in Figure 5.21.

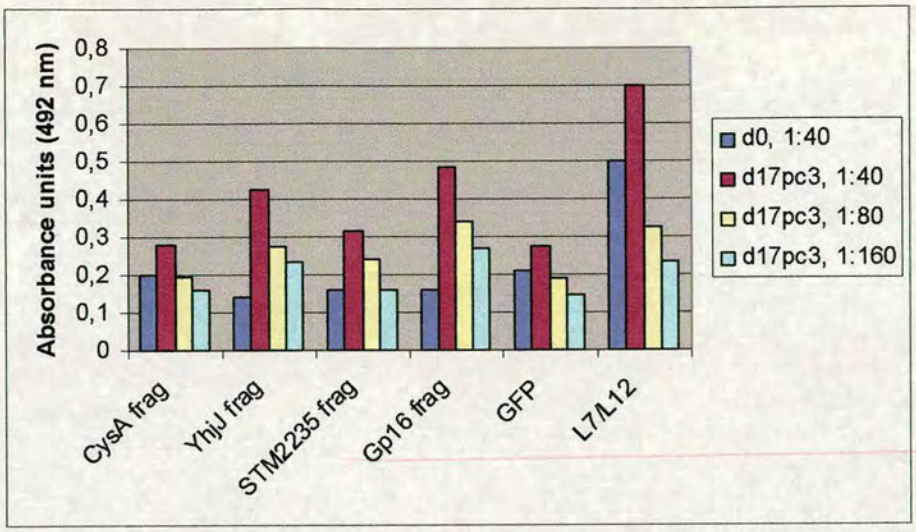


Figure 5.21. Relative absorbance values representing antigenicity of different 6× His-tagged constructs after testing by ELISA. Purified 6× His-tagged fragments of CysA, YhjJ, SMT2235, and Gp16 identified as potential antigens were tested with pre-immune (d0) and hyperimmune anti-*S.* Typhimurium (d17pc3) sera at the dilutions indicated. Positive and negative control antigens were L7/L12 and GFP, respectively.

To ensure that the low absorbance values obtained in the ELISA were not due to experimental error, a new positive control was added. GroEL is a well characterised *Salmonella* antigen (Panchanathan *et al.*, 1998; Dera-Tomaszewska *et al.*, 2003). For this reason, purified *S.* Typhimurium SL1344 GroEL (courtesy of P. Taylor: purified untagged protein (Taylor, 1997)) was likewise tested by ELISA in parallel with the potential antigens. Absorbance values, less the average value of secondary antibody control wells, are represented in Figure 5.22. Very high absorbance readings were produced by very dilute amounts of anti-*S.* Typhimurium serum in wells coated with GroEL. This occurred in parallel with very low readings produced by much higher preimmune serum concentrations in GroEL-coated wells. It was also in parallel with very low readings produced by high concentrations of both sera in wells coated with the other proteins.

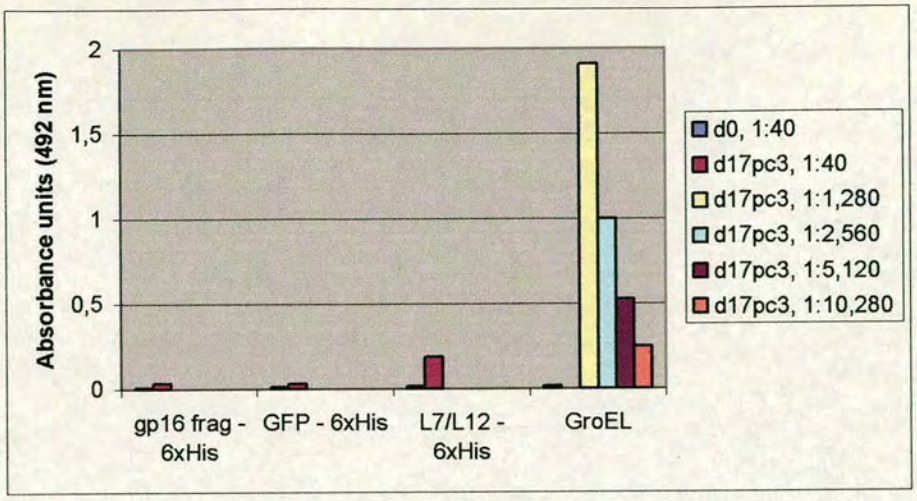


Figure 5.22. Relative absorbance values representing antigenicity of purified proteins after testing by ELISA. Purified 6× His-tagged L7/L12, GFP, and a fragment of Gp16 identified as potentially antigenic were tested with pre-immune (d0) and hyperimmune anti-*S.* Typhimurium (d17pc3) sera at the dilutions indicated. Untagged GroEL and 6× His-tagged GFP were positive and negative control antigens, respectively.

b. Western blots

Western blots were performed for comparison with the ELISA results. An SDS-PAGE gel was prepared, with the purified His-tagged constructs (0.2 µg per lane) alongside GroEL in duplicate, separated by a pre-stained molecular weight marker (New England Biolabs). After electrophoresis, proteins were transferred to PVDF membrane, after which the membrane was divided along the centre of the pre-stained marker. The two pieces of membrane were then blocked and subsequently probed with either HRP-conjugated His-probe H-3 mouse monoclonal antibody (Santa Cruz Biotechnology) at 1:1000 or hyperimmune anti-*S.* Typhimurium (d17pc3 CBA/Ca) serum at 1:4000, which was the same serum concentration used in the screening process. Protein bands were detected by ECL and photographed. The ECL detection and normally photographed PVDF membrane are shown (Figure

5.23). Figure 5.23(A) shows that GroEL (indicated with an arrow) reacts strongly with anti-*S.* Typhimurium serum and is accompanied by a weakly reactive contaminating band of approximately 16 kDa. L7/L12 (Figure 5.23(A), Lane 5) is weakly reactive with the same serum. The clearly transferred prestain marker, as well as the strongly reactive anti-histidine bands in Figure 5.23(C) confirm that an ample quantity of protein was transferred to the membrane for immunodetection purposes. Indeed, the three anti-histidine reactive bands present in the Gp16 frag preparation are present in very small quantities as seen in Lane 2 of Figure 5.20., with the smallest anti-histidine reactive band not being visible at all. The lack of anti-histidine reactivity of GroEL confirms that the antibody binding is specific. The major protein band present in the Gp16 frag preparation does not have the predicted molecular weight for this construct and is extremely faint in Figure 5.23(B). Thus, the major component of this preparation is likely to be a contaminant. The reason for multiple bands in Lanes 2 and 3 of Figure 5.23(B) is not clear but will be examined in the following discussion section of this chapter.

CysA frag is not included in Figure 5.23 because it had been completely consumed when this blot was performed. However, it was immunoblotted previously with anti-His antibody and also with hyperimmune anti-*S.* Typhimurium serum. ECL detected a single band, corresponding precisely to the band in Lane 1 of Figure 5.20, when the anti-his antibody was used. No bands were detected by ECL when the hyperimmune anti-*S.* Typhimurium serum was used (data not shown).

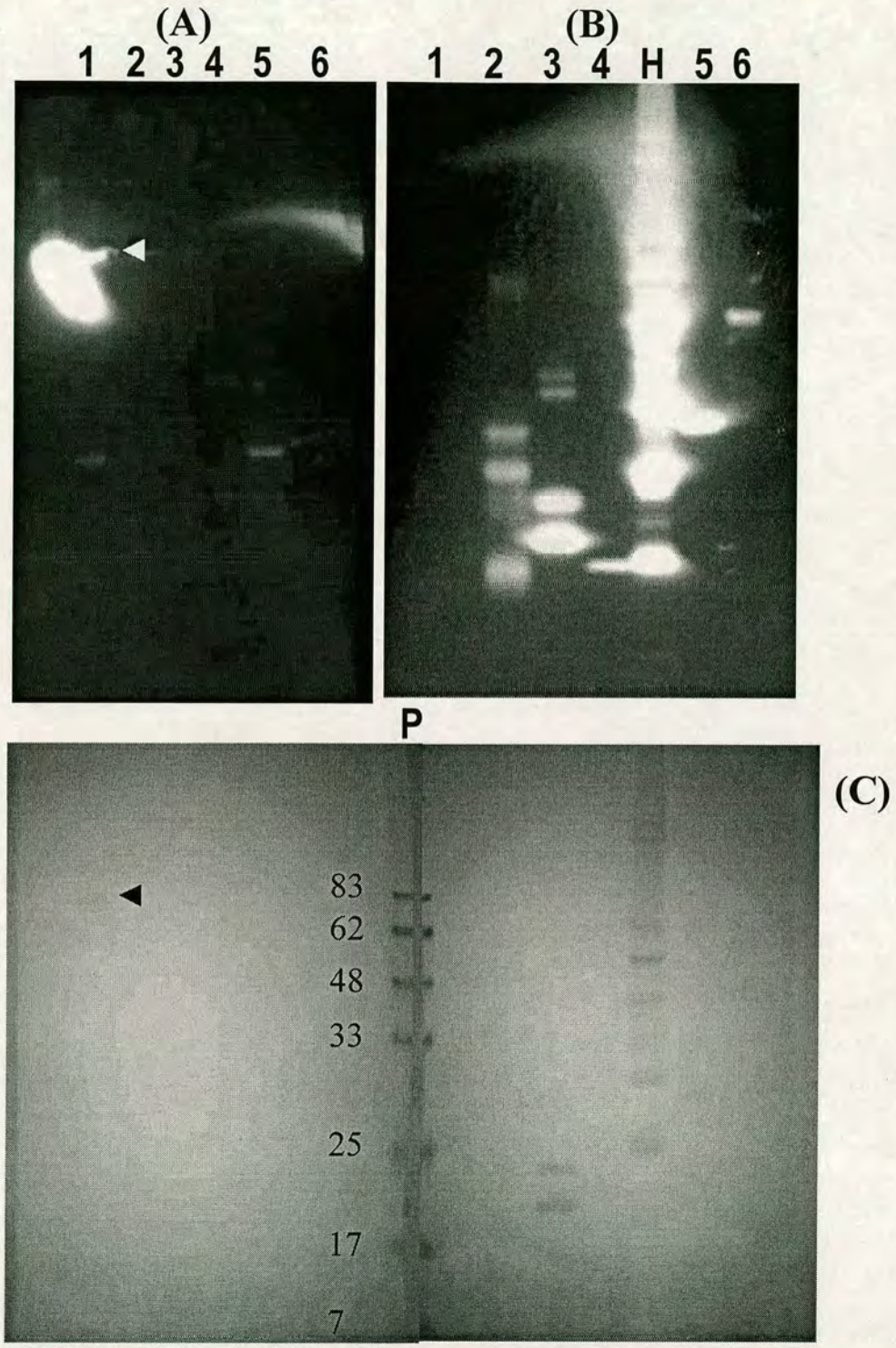


Figure 5.23. ECL detection of purified proteins, probed with (A) hyperimmune anti-*S.* Typhimurium serum or (B) anti-3×His antibody. (C) Divided PVDF membrane, after ECL, corresponding to the images directly above it. (0.2 µg each lane) Lane 1: GroEL; Lane 2: Gp16 frag-6×His; Lane 3: STM2235 frag-6×His; Lane 4: YhjJ frag-6×His; Lane 5: L7/L12-6×His; Lane 6: GFP-6×His; H: BenchMark His-tagged protein standard (Invitrogen). P: Broad-range prestained protein marker (New England Biolabs), with corresponding band sizes (in kDa) alongside the marker. Other protein bands visible in (C) appeared as a result of strongly reacting ECL components. The GroEL protein band is identified with an arrow.

C. Discussion

Following the identification of phagemid clones encoding potentially antigenic protein fragments in the previous chapter, the corresponding sequences were sub-cloned into expression vectors. IPTG-induced expression was detectable from positive (L7/L12) and negative (GFP) controls and from four of the ten clones in the conditions tested. Purification by affinity chromatography followed by IEC allowed the immunodetection of these protein fragment fusions by ELISA and western blot. The antigenicity of the four purified fusions was comparable to that of the negative control. Furthermore, the positive control was very weakly antigenic, as verified by a second positive control that was subsequently introduced.

1. Cloning

In order to match the chosen phagemid clones' sequences as closely as possible, protein fragments were cloned rather than entire proteins. This would reduce the possibility of an antigen being identified coincidentally with a phagemid clone. Ultimately, a phagemid clone isolated on the basis of antigenicity must encode an antigenic epitope. If a whole protein is tested for antigenicity, it may happen to be antigenic due to the presence of an epitope that is entirely absent from the phage-displayed portion. This would be no different than randomly testing proteins for antigenicity. The likelihood of coincidentally identifying an antigen should not be underestimated, as immunoblots in Chapter 3 suggested that the CBA/Ca mouse serum reacts with a variety of *S.* Typhimurium antigens. Additionally, coincidental

isolation of phagemid clones was highlighted in Chapter 4. The isolation of Clone 19 containing a portion of the gene encoding a well-known antigen, flagellin, was coincidental. The only evidence to prove its random presence was the minus orientation of the insert. If it had a plus orientation, it would not necessarily contain an antigenic epitope.

For sub-cloning the sequences encoding the protein fragments, TOPO and Gateway cloning were used for greater convenience. Cloning into the TOPO entry vector allows the flexibility to recombine the same insert sequence into different destination vectors to produce expression clones with different affinity tags (Invitrogen, 2003a). When choosing an affinity tag for purification of the target recombinant protein fragments, the polyhistidine tag was chosen primarily on the basis of its small size. Larger molecules such as GST could potentially mask the antigenicity of a small protein fragment. Cleavable affinity tags are also available. However, the efficiency of cleavage varies according to the recombinant protein to which the tag is fused (J. Bjerketorp, 2001, unpublished results). In the phagemid vector pG8SAET, phage-displayed protein fragments are fused to the small E-tag at the C-terminus. In order to more closely match the structure of phage-displayed protein fragments, a C-terminal His-tag fusion was chosen.

The nucleotide sequence of all constructs was checked to confirm that they did not contain mutations affecting the correct translation of the insert sequence. None of the constructs were mutated in any way. The *gp16* sequence obtained from Clone 122 deviated from the published sequence for the corresponding gene at two nucleotide positions, creating a difference at the amino acid level. The fact that the *gp16* expression clone had the identical sequence deviation from independently

amplified DNA indicates that this difference is related to the *S.* Typhimurium strain from which the DNA is amplified.

2. Expression

After extensive testing of induction conditions, only four of the ten protein fragment constructs showed detectable expression. It is possible that the other six expressed the recombinant fusion in the insoluble fraction, since the supernatant of cell sonicates were used for SDS-PAGE analysis. However, expression could not be detected in the lysates even with a monoclonal anti-3×His antibody for these six clones. This was despite the strongly detected expression of the *STM2235* construct, which is predicted to have a 95% chance of insolubility when overexpressed in *E. coli* (www.biotech.ou.edu). Among all the constructs, this one had the highest probability of insolubility as predicted by the amino acid sequence. Thus, if expression had occurred in the other constructs, it should have been detectable in the supernatant after sonication with the anti-3×His antibody. The failed attempt to enrich for possibly expressed fusions from the six by performing Ni-NTA affinity chromatography on whole cell lysates supported the conclusion that there was no expressed fusion to purify from these six. However, a better assessment could be performed on denatured lysates in which inclusion bodies are disrupted by guanidine or urea (Kuliopulos, 1994).

Additionally, the *STM2235* construct is predicted to be the least stable protein among all the constructs according to the "N-end rule" because it is the only one encoding Met followed by Leu in positions one and two of the amino acid sequence.

Processing of the N-formyl methionine exposes leucine at the N-terminus, leading to a significantly decreased half-life. This instability is dependent on the activity of the Clp protease in *E. coli* (Tobias *et al.*, 1991). Although *E. coli* BL21 is naturally deficient in Lon and OmpT proteases, Clp deficiency has not been described for this strain. If instability played a role in the lack of expression for the six clones, it was not instability related to the N-end rule.

Many other degradation pathways exist in *E. coli*, including a protease that specifically targets small (<15 kDa) proteins (Kim *et al.*, 1995). Instability of small proteins can be counteracted by reducing growth temperature, inducing for a shorter time, and/or using a dihydrofolate reductase (DHFR) tag (Qiagen, 2003). Expression levels were not investigated at 1 hr after addition of IPTG with growth at 30°C, and this could be a reason for the lack of observable expression from the six constructs.

An alternative system for the expression of recombinant peptides is by N-terminal fusion to ketosteroid isomerase (KSI) in conjunction with C-terminal fusion to a polyhistidine tag. KSI is highly insoluble and is rapidly directed to inclusion bodies when produced. This protects fused peptides from degradation and improves the yield. After affinity purification of inclusion bodies under denaturing conditions, the recombinant peptide is released from KSI and the polyhistidine tag by CNBr cleavage of junctional methionines (Kuliopulos and Walsh, 1994). Protein fragments may be expressed more stably when fused to larger affinity tags. Native-purified protein fragments fused to maltose-binding protein (MBP) have been successfully used in the analysis of domain interactions of the fusion-active complex of SARS virus (Ingallinella *et al.*, 2004). A GST tag fused to antigenic fragments of

Chlamydia abortus outer membrane proteins enabled affinity purification and also did not interfere with antibody binding (Vretou *et al.*, 2003).

3. Purification

The purity of a tagged fusion protein after affinity chromatography is in part dependent on expression levels (Ramage *et al.*, 2002). Thus, it is not surprising that the purest eluted protein after affinity chromatography in native conditions was the L7/L12 construct, which expressed the most fusion protein relative to other cellular proteins. Conversely, native Ni-NTA affinity chromatography performed on lysates of the four sub-clones with very low expression levels yielded fusion proteins of very low purity. A second step in purification was thus introduced.

IEC, which is frequently used as the last step to yield highly pure protein preparations for crystallography (Ramage *et al.*, 2002), greatly enhanced the purity of all eluted proteins. Additionally, successful purification of biologically active polyhistidine-tagged fragments of fibronectin by IEC following Ni-NTA affinity chromatography has been described (Klein *et al.*, 2003). Except in the case of STM2235 frag, IEC eluted protein fractions containing the target protein were sufficiently pure to use for immunodetection. Except in the case of Gp16 frag and the second attempted STM2235 frag IEC purification, all of the target proteins bound to the column. A lack of binding to the IEC column was accompanied by an attempt to concentrate the sample, using PEG and dialysis tubing, prior to loading it onto the column. Significantly, it was assumed that the molecular weight cutoff of 3.5 kDa would guarantee that the fusions would not pass through it. This was not verified,

and drew further suspicion after the final Gp16 frag preparation was concentrated using this method. The protein band corresponding to Gp16 frag his was extremely faint after PEG-induced extraction of buffer through dialysis membrane, and a larger contaminating band became very concentrated.

Due to a shortage of time to perform further experiments, denaturing conditions were used when fusion protein had to be newly purified from cell lysate. Native conditions should be used whenever possible to ensure the correct folding of a protein, which is important for detecting some antigenic epitopes (Somner *et al.*, 1999). Although the conformation of polyhistidine-tagged protein fragments may be different from the corresponding E-tagged fragments displayed on the phage surface, it is prudent to retain native conformation whenever possible since it is also possible that the conformations may be the same. However, a refolding buffer can be used in conjunction with denaturing conditions to partially compensate for a loss of conformation (Tsumoto *et al.*, 2003). This was a convenient solution since binding to Ni-NTA in denaturing conditions yielded much purer fusion protein after elution with the refolding buffer. This phenomenon is likely due to *E. coli* metalloproteins which co-purify on Ni-NTA in native conditions (Pasquinelli *et al.*, 2000) that lose the metal-binding site due to disrupted protein structure in denaturing conditions. Since high purity was important for minimising the possibility of the polyclonal anti-*S.* Typhimurium serum reacting with impurities originating from the closely related *E. coli*, and shorter amino acid sequences are more flexible in conformation, this was considered the best strategy to complete the purification scheme.

GFP, L7/L12, CysA frag, and YhjJ frag fusions were highly pure after Ni-NTA and IEC separation from host cell proteins, characterised by a single band

observed in SDS-PAGE analysis (Figure 5.20) that correlated with a single anti-polyhistidine reactive band in western blots (Figure 5.23(C), data not shown). Only Gp16 frag and STM2235 frag preparations contained multiple, anti-polyhistidine reactive bands. The band of highest molecular weight in the STM2235 frag preparation corresponds in size to a possible dimer. Although the sample was prepared with DTT prior to electrophoresis, oxidation caused by presence of leached nickel ions from the Ni-NTA could counteract its effect. Indeed, leaching of nickel ions during affinity purification has led to oxidation of the purified polyhistidine-tagged construct in other studies (Ramage *et al.*, 2002). However, this explanation cannot be applied to the other bands in the two protein fragment preparations. Significantly, both of these constructs are derived from bacteriophage genes. Multifunctional proteins are not uncommon among phages and viruses (Ray and Ray, 2001; Zhao *et al.*, 2001; Toth *et al.*, 2003). Furthermore, multiple distinct polypeptides may be produced by a single viral gene, according to different translation mechanisms (Vassilaki and Mavromara, 2003; Zimmer *et al.*, 2003). New polypeptide forms of a viral gene may be detectable by expression of C-terminal fusion tags (N. Vassilaki, personal communication). The production of multiple proteins from a single sequence, and multifunctional characteristics of some viral and phage proteins, may be the product of evolutionary pressure to maintain a small genome size (Yewdell and Hill, 2002).

4. Immunodetection

Only four of the ten constructs could be tested for antigenicity. ELISA and western blots failed to identify any of these as antigens. Surprisingly, the intended positive control, L7/L12, was an extremely weak antigen for the CBA/Ca mice in which the hyperimmune serum was produced. It was barely detectable in all ELISAs and western blots, although the serum strongly reacted with a known antigen, GroEL, in parallel. This is in contrast to hyperimmune anti-*S.* Typhimurium serum prepared in the same manner in BALB/c mice (M. Mogensen, 2004, unpublished results). Since these two strains of inbred mice have different H-2 haplotypes ($H\text{-}2^d$ for BALB/c and $H\text{-}2^k$ for CBA/Ca) they recognise different antigenic peptides. This could explain why L7/L12 is strongly antigenic for BALB/c mice but not for CBA/Ca mice. Indeed, genetic differences may explain why immunoblots detected L7/L12 as an antigen recognised by serum from some *Chlamydia trachomatis*-infected individuals but not others (Sanchez-Campillo *et al.*, 1999).

None of the subcloned constructs could be demonstrated as antigenic. A sufficiently pure and detectable amount of the expressed fusion constructs was used for antigenicity testing, except in the case of Gp16 frag. The three polyhistidine-tagged forms visible in Figure 5.23(C), Lane 2, may not be present in a high enough concentration for reliable ELISA testing. An ELISA test using the anti-polyhistidine antibody would clarify the detectability of all the fusion proteins bound to the plate. Furthermore, the possibility of conformational antigenic epitopes being present among the sub-cloned fragments cannot be ruled out, since denaturing conditions were employed in the purification of three out of four. If Gp16 frag is antigenic, the

different forms would have to be isolated by other purification methods so that each can be independently tested for antigenicity. The possibility of this protein's antigenicity draws attention to its possible expression during the course of murine infection and how this may be related to pathogenesis. As pointed out in Chapter 1, bacteriophage production during the course of pathogenesis has been documented, although not yet in the case of *Salmonella*.

The magnitude of difference in antigenicity between GroEL and L7/L12, as well as the lack of antigenicity exhibited by the other fusions, was consistent between the immunoblot and ELISA results. The antigenic potential of any of the constructs cannot be ruled out; however, the intensity with which the phage-displayed fragments give a positive result in a dot blot (Figure 4.5, Chapter 4), relative to phage-displayed L7/L12 --now established as very weakly antigenic--, does not support the likelihood of strong antigenicity among any of the ten selected clones.

Since the protein fragments could not be proven as antigenic, it is possible that the fragments displayed by the ten clones attracted the binding of *E. coli* chaperone proteins. GroEL, shown above as strongly recognised by anti-*S.* Typhimurium serum, is part of the HSP60 family of proteins that are involved in the folding and refolding of polypeptides (Craig *et al.*, 1993). This protein is highly conserved, being nearly identical for *E. coli* and *Salmonella* (Lillicrap *et al.*, 2004). GroEL binds to a large variety of sequences that it recognises as being in an abnormal conformation (Chatellier *et al.*, 1999; Kandror *et al.*, 1999). Since the ten clones are clearly distinguishable from the negative control in Figure 4.5 (Chapter 4), GroEL could be binding, in small quantities, to protein fragments on the surface of

the particles produced by the ten phagemid clones. Since the negative control is not expected to display any protein fragments, it would not attract the binding of this chaperone. To assess whether host GroEL is binding to the phage particles, the same dot blots could be performed using commercially available anti-GroEL antibody.

Purification of the protein fragments was important for clarifying the reasons for the positive results observed in Chapter 4. Thus, the results obtained in this chapter not only reveal that a different positive control antigen is necessary, but also that the screening procedure should be modified to reduce the likelihood of misleading results arising from possible binding of host GroEL or other proteins. These and biopanning-related modifications will be discussed further in the following chapter.

CHAPTER 6

Summary and Future Possibilities

A. Summary

Emerging antibiotic resistance has drawn new attention to vaccine development (Graham, 2002). Two vaccines are currently licensed for typhoid fever in humans: a live attenuated strain that is not suitable for immunocompromised individuals, and a polysaccharide subunit vaccine whose antigen is produced by a segment of the *S.* Typhi genome that is unstable (Ivanoff *et al.*, 1994; Plotkin and Bouveret-Le Cam, 1995; Nair *et al.*, 2004). Vaccination with multiple antigenic subunits offers an ideal solution to this problem. *S.* Typhimurium infection of mice has been a useful model for the study of typhoid fever (Carter and Collins, 1974; Jones and Falkow, 1996). Furthermore, immunoblot patterns of mouse serum against *S.* Typhimurium lysate closely resemble those of human serum against *S.* Typhi lysate (Brown and Hormaeche, 1989).

Using the mouse model, the viability of multiple subunit vaccination was recently shown. Briefly, a promoter trap library derived from *S.* Typhimurium DNA was constructed upstream of plasmid-encoded GFP-ovalbumin. BALB/c mice were infected with *S.* Typhimurium bearing the library, after which genes associated with increased promoter activity were identified. Corresponding proteins were purified and used in conjunction with Freund's complete and incomplete adjuvant in primary and secondary immunisations, respectively (Rollenhagen *et al.*, 2004). The method used to identify the antigens was laborious and time-consuming, with the antigenicity of the proteins being inferred from *in vivo* expression levels. Freund's adjuvant is standardly used in animals, but its side effects are unacceptably severe for use in humans. The protection level achieved was high and comparable to that conferred by

a whole-cell lysate of the pathogen. However, the protective subunit vaccine consisted of only two antigens, which may not produce the same result if used to immunise mouse strains other than BALB/c. This is significant in light of the need for a vaccine suitable for a genetically diverse population with varied responses to specific antigens. Moreover, the use of multiple antigens will reduce the likelihood of antigenic drift, such as may be happening for the currently used Vi subunit vaccine (Nair *et al.*, 2004).

The objective of this thesis was to identify *S.* Typhimurium antigens by phage display technology. A phagemid library was constructed using *S.* Typhimurium DNA and hyperimmune *S.* Typhimurium-reactive serum was raised in mice. With these tools, a series of selection protocols (biopannings) were tested and modified until a successful biopanning was inferred. A number of clones were screened for possible antigenicity against a constructed phagemid clone that was considered as the positive control. This screening process led to the selection of a final ten phagemid clones encoding protein fragments. The corresponding gene fragments were amplified from *S.* Typhimurium DNA and cloned into an expression vector encoding an affinity tag for purification. Expression and purification was achieved for four of these, which were subsequently tested for antigenicity. Antigenicity could not be demonstrated for any of the four, and the protein that was thought to be antigenic was demonstrated as very weakly antigenic. Hence, while it is possible that the purified protein fragments did not display the same epitopes as when bound to the phage, it is more likely that the biopanning was not successful and an inappropriate protein was chosen as the positive control for screening. Although the main aim of this work was not achieved, much can be learnt from the present study to allow the successful use of this

technology in the future. The phagemid library and serum used for biopanning were demonstrated to be of a high quality and are the most important tools for future experiments, which are discussed below.

B. New biopanning strategies

1. New control biopannings

The majority of biopanning studies are performed with ligands of maximal homogeneity, such as a single protein, a monoclonal antibody, or purified fractions of serum (Theisen *et al.*, 2000; Kouzmitcheva *et al.*, 2001; Robben *et al.*, 2002). It is for this reason that IgG was partially purified from the hyperimmune anti-*S.* Typhimurium serum by ammonium sulphate precipitation. However, there was no test to confirm whether the level of purification was sufficient for successful biopanning. In order to prove that a successful biopanning is achievable with the pG8SAET *S.* Typhimurium library, a homogenous ligand should be used in an initial control biopanning. The recent analyses of the *S.* Typhimurium ShdA protein as a fibronectin-binding protein (Kingsley *et al.*, 2002; Kingsley *et al.*, 2004) make this interaction a convenient tool for biopanning experiments. Since the target protein is already known, a successful biopanning of the phagemid library against fibronectin will be obvious. Therefore, the conditions of this experiment could be more easily adjusted to achieve optimisation, which would provide useful information for subsequent biopannings against other targets. Ideally, the next ligand should be a monoclonal antibody directed against the same protein, since its detectability from

the library will have been established. Biopanning against this ligand could be optimised in turn, and the monoclonal antibody could be diluted in increasing proportions of mouse antibody preparations, until a limit of detection is reached. The optimal conditions for a successful biopanning at this limit would then be used for biopanning against successive purifications of hyperimmune anti-*S.* typhimurium serum. This sequence of experiments will provide a stronger foundation for inferring a positive result, thus meriting the further screening of clones and the testing of candidate antigens.

A second option for a new biopanning strategy would be to raise new hyperimmune serum directed against a single antigen, such as OmpC, flagellin, or AhpC. Samples of successive purifications could be used as ligands for biopanning, beginning with the most pure and optimising for each step of decreasing ligand purity. This would have the advantage of mimicking a hyperimmune anti-*S.* Typhimurium serum biopanning more closely. However, the most pure form of this antibody preparation is likely to be less pure as a ligand in comparison to fibronectin. Alternatively, monoclonal antibodies could be raised against these proteins and used for biopanning trials. The epitope to which monoclonal antibody binds within the OmpA protein has been defined, so this antigen-antibody pair could be exploited for trial biopannings (Singh *et al.*, 2003).

2. Suggested modifications for improved selection and screening

As mentioned above, a successful biopanning has yet to be demonstrated using the phagemid library prepared in Chapter 3. If trial biopannings are performed against a homogenous ligand such as fibronectin, it may become clear whether proteolytic degradation of displayed fragments has caused a decrease in the efficiency of ligand binding. While this is a more crucial point for gene III-based systems than for geneVIII due to the difference in copy number (Baek *et al.*, 2002), it may be a contributing factor and thus should be investigated for this library. Jacobsson *et al.* (2001) and many others did not need to use protease inhibitors for successful enrichment, but this could have been compensated by the homogeneity of ligand. If proteolysis is suspected to interfere with biopanning, the phagemid library could be re-infected with R408, and particles could be extracted in the presence of protease inhibitors. To further enhance the stability of displayed protein fragments, glycerol also could be included in the biopanning solutions. This has been demonstrated to enhance binding specificity during selection (Kjaer *et al.*, 1998). One possibility that has not been assessed is whether interactions between phage particles could interfere with binding. Tween 20 was used in the washing solution for removal of non-specifically binding phage. The same component could be added to the library prior to panning, to reduce the frequency of any phage-phage complexes that could be masking antigenic epitiopes.

Since successive purification of antibody from serum is somewhat difficult when very small quantities are available, such as from mice, a capture method may be used in combination with purification. An anti-mouse antibody was used during

initial pannings, which led to enrichment of phage binding to the anti-mouse antibody. A more appropriate capture method may be to use a non-antibody protein such as protein G. This protein binds to the Fc and Fab regions of the antibody molecule, as well as to albumin. However, a mutant form lacking the Fab and albumin binding regions could be used (Goward *et al.*, 1990). Mutant protein G could be coated onto microwells in order to capture the mouse IgG at the Fc terminus, increasing the efficiency of selection by ensuring that the antigen-binding region is exposed to the phage library. If background binding to protein G interferes with enrichment of phage encoding antigens, protein G and anti-mouse antibody could be used in the first and second rounds of biopanning, respectively.

The possibility of the hyperimmune anti-*S.* Typhimurium serum reacting with host components, such as GroEL, highlights the need for elimination of antibodies with undesirable binding from the serum. Kouzmitcheva *et al.* (2001) depleted their IgG preparation of antibodies that may bind phage non-specifically by pre-adsorbing the ligand with inactivated wild-type phage several times. This could be combined with pre-adsorption of the serum with a lysate of host cells. Indeed, the elimination of human antibodies binding to host cell and phage components was found to be crucial to the identification of tumor antigens (Chen, 2004).

Another way to reduce the proportion of undesirable phage recovered from a round of selection involves a stepwise reduction in pH during elution (D'Mello and Howard, 2001). This is reminiscent of the salt gradient used to elute target proteins during IEC purification (Chapter 5). In fact, the use of 2 M NaCl in one study enabled the isolation of a clone that was not recovered by elution with low pH (Jacobsson *et al.*, 2003). This study tested mild and harsh elution conditions: 5%

Tween, 2 M NaCl, 5 M urea, 40% ethanol, 80% methanol, 5% Tween, and 40% ethylene glycol. Low pH, not 2 M NaCl, was determined as the most efficient eluting agent. Thus, the specific nature of binding between a particle and the ligand determine which conditions are necessary for disrupting that interaction. Indeed, chemical elution is highly dependent on the specific antigen-antibody pair in question (Schier *et al.*, 1996; de Bruin *et al.*, 1999). More recently, an affinity-independent method for elution was tested. Streptavidin, attached to a solid support by a DNA linker, can be detached by the addition of nuclease in the Cellection Biotin Binder Kit (Dynal). After panning the biotinylated ligand complexed with streptavidin, the phage-ligand complexes are eluted from the solid support by degradation of the DNA linker. This method solves the problem of poor recovery of clones with very high binding affinity (Santala and Saviranta, 2004). Thus, biotinylated mutant protein G could be used for biopanning in this manner.

For subsequent screening of clones recovered from biopanning against hyperimmune anti-*S.* Typhimurium serum, a suitable positive control will be needed. A fragment of OmpC containing a known antigenic region could be cloned into the vector (Singh *et al.*, 1995). Clearly, antigenicity of the fragment should not be assumed under any circumstances and should be verified prior to its use as a positive control with serum from any given mouse strain. The strikingly weak antigenicity of L7/L12 in CBA/Ca mice, compared to BALB/c, highlighted an important point. The variability between mouse strains in the antigens they recognise should be addressed when biopanning, as it is relevant for eventual vaccine development. Hyperimmune serum from more than one strain of mouse should be used to compensate for the variability of antigen recognition associated with differing H-2 haplotypes. A study

that successfully identified mimotopes for diagnosis of infection with *Borrelia burgdorferi* (Kouzmitcheva *et al.*, 2001) used antibody from one individual for the first round of biopanning, antibody from another individual in the second round, and a mixture of both for a third round. A similar strategy could be used for BALB/c and CBA/Ca antibody preparations. Alternatively, serum could be raised in heterozygote and/or outbred mice to more closely mimic a heterozygous population.

C. Immunisation Strategies: Direct Immunisation With Phage

There are even greater benefits to be gained from phage display than the identification of antigenic epitopes. Animals have been directly immunised with recombinant phage which elicited antibodies against the antigen that was mimicked by the displayed peptide (Zuercher *et al.*, 2000). Since the viral particles themselves are highly immunogenic, adjuvant is not needed (Greenwood *et al.*, 1991). In addition, recombinant phage can elicit both humoral and cell-mediated immunity (Heal *et al.*, 1999; Manoutcharian *et al.*, 1999). An immune response can be generated by peptides fused to the coat proteins encoded by gene VIII or gene III (Yip *et al.*, 2001), and recombinant phage have been used as part of sucessful intradermal anti-cysticercosis and oral anti-IgE immunisations in mice (Manoutcharian *et al.*, 1999; Zuercher *et al.*, 2000).

A vaccine consisting of a defined set of several epitopes could elicit protection without the risk of causing disease. It is important that several epitopes be used because the specificity of antibodies elicited to a particular disease will vary from one individual to the next (Germaschewski and Murray, 1996). Vaccinating

with recombinant phage would be better than with protein plus adjuvant for several reasons. Firstly, adjuvant would not be needed if phage are used. Secondly, a phage vaccine would be cheaper and easier to produce. Thirdly, phage elicit both humoral and cell-mediated immunity, which is particularly relevant for *Salmonella* infection due to its intracellular localisation. Such recombinant phage can be used to directly immunise against the antigen of choice. A major disadvantage would arise from the induction of antibodies to the phage itself, and this might interfere with future vaccinations for a different disease where the same phage is used as the vector for immunisation.

D. Phage Display and *S.* Typhimurium: Concluding Remarks

Phage display offers many advantages for identifying antigens and serving as a carrier for these antigens in immunisation. Many parameters affect the copy number and stability of displayed protein fragments, and non-specific binding can interfere with the isolation of specifically binding particles. However, optimisation of selection procedures can overcome these problems to generate valuable results for the future design of multiple subunit vaccines. Following the selection of appropriate antigens for use in such vaccines, different immunisation protocols should be tested in order to determine efficacy of protection. Furthermore, different strains of mice should be used in these protocols to infer how broadly useful the antigens may be in a genetically diverse population.

APPENDIX:

5'-3' Nucleotide Sequences of Clones Represented in Table 4.9 with Corresponding Predicted Amino Acid Sequences

THE SIX CONSECUTIVE AMINO ACIDS WITH THE HIGHEST ANTIGENICITY FOR EACH CLONE ARE INDICATED IN BOLD

1. GGACGGCATTTCCCGGCACGAATACGACTTAGCCCTTGCCAGG
(**DGISRH**EYDLALAR)

2.
GCCAGAAAGTGAACGTGGGCGATACCCTGTGCATCGTTGAAGCCATGAAAATGATGAACCAGATCGAA
GCTGACAAAGCAGGTACTGTGAAAGCGATTCTGGTCGAAAGTGGTCAACCGGTAGAATTTGACGAGCC
ACTGGTCGTCATC
(QKVNVGDTLCIVEAMKMMNQIEADKAGTVKAILVE**SGQPVE**FDEPLVVI)

3.
CGTACGACGCCGACTTAGATTAGTCGGCTTTTTTTTGCCTGTAGACTATCTGCGTCTACCCTTTAAGA
GTCTGAATGACATTCTGGAGGGTAATATGAGCTGTCTTTTGACCTTATTATATCTACACTCGTCCTTG
TCGGACCCGATTCCCACTGACCCTGTTCCCATTCCTGAGCCGCTTCCCCGTCCTCAGCCGATGCCCGA
CCCGCCGCCGGAC
(VRRRLRLVGFFLPVDYLRLPFKSLNDILEGNMSCLLTLLYLHSSLSDPIPTDPVPIPE**PLPRPQ**PMP
DPPPD)

4.
TTGACATCGGTAACTCATCAACGGAAGTCGCACTGGCGCGGCAAGATGAGACTGGCGCACTGACGATT
ACACACAGCGCGCTGGCGGAAACCACCGGGATCAAAGGCACGTTGCGTAACGTGTTCGGCATTCAGGA
AGCGCTCGCCCTCGTCGCAAAGCGCGCGGGGATCAATGTCAGAGATATTTCGCTCATCCGCATTAACG
AAGCCACGCCGGTGATTGGCGATGTGGCGATGGAAACCATTACCGAAACCATCATCACCGAATCGACA
ATGATCGGCCATAACCCAAAAACGCCGGGCGGAGCAGGCCTTGGTGTGGGTATCACGATTACGCCGGA
GGAGCTG
(DIGNSSTEVALARQDETGALTITHSALAETTGIKGTLRNVFGIQEALALVAKRAGINVRDISLIRIN
EATPVIGDVAMETITETIITESTMIGHN**PKTPGG**AGLGVGITITPEEL)

5. CCTGCAGCTCCACAAATAGCATGGCGTGTAG
(LQLH**KWHGVW**)

6.
TCTCGTAGGCTATCATGGAGGCACAGCGGCGGCAATCCCGACCCTACTTTGTAGGGGAGGGCGCACTT
ACCGGTTTCTCTTCGAGAAACTGGCCTAACGGCCACCCTTCGGGCGGTGCGCTCTCC
(LVGYHGGTAAAIPTLLC**RGGRTY**RFLFEKLAWRPPFGRCAL)

7.
CAGCCGCCGCTGCCACACGTACAGGTAGCGTGCTGCGTCTGTTGATCTCTGGTCCGCTGGCTTTGCTG
CGCGTGGCATTATTTGCTGTTGGTAGCCTGCTGGGTGCGCTGCTCAGTCCTGTAGGGCTGGTTGTGGC
TGCACTGGCAGGCGTGGCGCTGGTTATCTGGAAATACTGGCAGCCCATCAGTGCGTTTCTGGGGGGCG
TGGTGGAAGGGTTCAAAGCCGCTGCTGCGCCCATCAGCGCCGCCTTTGAGCCGCTCAGACCCGTGTTT
CAGTGGATTGGTGACAGGGTGCAGGCCTTGTGGGGCTGGTTCAATGATTTACTTACCCCGGTTAAATC
CACTGCCGAAGAACTGAACAGCGCAGCTGCAATG
(AAA**ATRTGS**VLRLLISGPLALLRVALFAVGSLLGALLSPVGLVVAALAGVALVIWKYWQPISAFLGG
VVEGFKAAAAPISAAFEPLRPVFQWIGDRVQALWGWFNDLLTPVKSTAEELNSAAAM)

8.
ACCGAAGTGATCGTTAACGTGGGCGGCACCATGCAGATGCTCGGTCGCCACAACAGCCAGCCACAGCA
GGAGCCTCAGACCCCACCT
(TEVIVNVGGTMQMLGRHNSQ**PQQEPQ**TPP)

9.
GGATTCTCCTCTACCAGTACAGGTCATTGAGGCCAGCCCGAAAGGTCACTATACACAGTTAGTGGTAC
AGCCGCTGGGGTGGTATCACGATCCGCTG
(DSPLPVQVIE**ASPKGH**YTQLVVQPLGWYHDPL)

10.
CACTGGACGTGGTGCGCCATAATCCTGATAGCTTCCGCGTTATCGCGCTGGTGGCGGGTAAAAATGTC
GCCCGCATGGCGGAGCAGTGCCTGGAGTTCTCGCCGCGTTATGCCGTGATGGACGATACGTCGAGCGC
AGAACAGCTCAAA
(LDVVR**HNPDSF**RVIALVAGKNVARMAEQCLEFSPRYAVMDDTSSAEQLK)

11.
CTATTGTCAGTGGGTTACGAACCCCCTTCGCTCGTCAGGCCACTGCATTTCATGGTATTCCCGCTGTT
GATCTCGGCAAGATGGTGGTAG
(LLSVGY**EPPSLV**RPLHFMVFPLLISARWWW)

12.
GCGGCCATAGTGGCGGCTGTCGGCGCTGGCCGGGTCGGCGTCGTACTCGCTGGCCAGCGTCCGGGCA
(**RPWWRL**SALAGSASYSLASVRA)

13.
CCGGCACCATCAGCACGCCGCGCGCTTTCAAGCGTTGATAGAGTAACTCGGTGGTAATCGGCAGATCT
TTAAACCACAGCCACAAAAATATTGCGCCTTCTGGTTTATGAATCAGGCAGCGTTCTTCGGATAAATA
GCGACGAATGATCGCGATCGTCTGTTGAACGCGCTGATAGTAGAAAGGTTTGATAACCGTTTCCGACA
GCCGCAGCAGATCGTTACGCTTAATCATTTCGCACATCATCGCCGGGCCCATGCCCCCGGGCGCAAGG
CTGATGATGCCGTTCATGTTGGCAATGGCGGTAATCGTCTTATCATTGGCGATGATAATCCC
(NO ORF)

14.
CAATTTAATATTGTATTATTTTCCTCTACTCTGGCTCTATGCTTGTTTGTCATGTTCACAAAAGGTGT
TAGTTTTAGATTTTCAGACACCCTAAAGCAAGTCGTTGAGTATCGTATGGACAACTCTCCCTGGAGGC
CTGATATTTGCTTCCTCAATCCAGATCAAGATTATTCAGCATTCTCAAAATGTCAGGATAAAATGACT
GAAAAG
(QFNIVLFSSTLALCLFVMFTKGVSFRFSDTLKQVVEY**RMDNSP**WRPDICFLNPDQDYSAFSKCQDKM
TEK)

15. GAATGACACGTAAACCGCGCAGCGCGCAGTCGCGGGCGATCCCTGCGCCTGTT
(MTR**KPRSAQ**SRAIPAPV)

16.
ATCTACACTTTTTAACTGTAACCACTCTGTTTATGAATTATTGCAAGATTCTCTGCTGCGTTAACCCG
CGGCGGCGAACGCTTTTTATCCCTTATTTGAGGATTTGACTGACACGTGCACTGTTGGAAGAGGTTAT
CCGACATATCCACCATAACAGGAGCATCTTATGAAAATGACAAAACTGACTACGCTTTTACTGACCGC
TACCCTCGGCCTTGCCAGCGGCGCAGCGTTAGCT
(NO ORF)

17.
CTGATGGATTCATCAGATATGACGCCGGAAGACTGGCAGCATATCGCTGCAGATATCAAAGCGCACTA
CGATGAGTATGACGGTTTTGTTATTCTGCACGGCACCGACACGATGGCGTTTACCGCATCGGCGCTCT
CTTTCATGCTGGAAAATCTGGGTAAACCGGTCATTGTGACAGGGTCACAAATTCCGCTGGCTGAGCTG
CGCTCCGACGGGCAAATTAATTTGCTGAATGCGCTTTATGTCGCCGCCAACTACCCTATTAATGAAGT
AACGCTGTTTTTTAATAATCGTCTGTTCCGCGGTAATCGCACGACCAAAGCCCATGCGGATGGGTTTG
ACGCTTTTGCCTCTCCCAATCTGCCGCCCTTGCTGGAAGCAGGAATTCACATTCGCCGTTTAAATACG
CCTCCCGCGCCCTACGGTTTTGGTGAATTAATCGTTCACCCTATTACGCCGCAGCCGATAGGCGTAGT
GACGATTTATCCGGGGATTTCCGCCGAGGTAGTACGTAACTTTCTGCGTCAGCCGGTAAAAGCGCTGA
TTCTGCGCTCTTATGGCGTAGGCAACGCGCCGCAAAATAAAGCGTTTTTACAGGAGCTGAAAGAGGCC
AGTTCGCGCGGCATCGTGGTGGTTAACCTGACGCAATGCATGTCAGGGCGAGTCAATATGGGCGGGTA
CGCTACCGGTAATGCGCTGGCGCACGCTGGCGTGATTGGCGGTGCCGATATGACCGTCGAAGCC

(LMDSSDMTPEDWQHIAADIKAHYDEYDGFVILHGTDTMAFTASALSFMLENLGKPVIVTGSQIPLAE
LRSDGQINLLNALYVAANYPINEVTLFFNNRLFRGNRTTKAHADGFDAFASPNLPPLLEAGIHIRRLN
TPPAPYGFGELIVHPITPQPIGVVTIYPGISAEVVRNFLRQPVKALILRSYGVGNAPQNKAFLQELKE
ASSRGIVVVNLTQCMSGRVNMGGYATGNALAHAGVIGGADMTVEA)

18.
CCGCCGGTAATGGTAATGAAGCCATCAAGCCCGTCGCGTTCGGCGTGGTCGAACAACACAATACCGCG
GCTGACGTTACGACCGCTGGGATCGTCATCGCTGGCCACCAGCGGGCGGACGCCGGAATACGCGCGCA
GAATACGCGTCTTCGCCATGACAGGGGCCAGTTTTTCCCCTTCACGCAGCAGGATATCGACTTCATCT
GCGGTGACGCGGTTGCTGTCTATCTCATTGTAATCAATATGGGTAGAGGTGGTGCCGATCAGTGAAAT
CGTATCGCCAGGTACCAGAATGTCGGCATCCGATGGTTTCCGGCAGCGGTTAATGACATGCTGGTTGA
TCCGATGGTCCATGATCAACAGCGAGCCTTTCGCCGGGAACATGCGAATACTCAGGTCGGCGTATTCG
GCGATCTGTTGCCCCCAGATACCTGCCG
(NO ORF)

19.
GAGTTGATCTGCTTCAGATCGATATCGATAGTTTCACCGTCGTTGGCACCAACCTGGATGGTCAGGGT
GTTGTCCTGCGCCAGGACTTTCACGCCGTTGAACTGAGTCTGGCCGGATACACGGTCGATTTCGTTCA
GGCGCTGGGTGATTTCAGCCTGGATGGAGTCGAGGTCAGACTGGGAGTTGGTGCTGTTAGCAGACTGA
ACCGCCAGTTCACG
(VDLLQIDIDSFTVVGT**NLDGQG**VVLRQDFHAVELSLAGYTVDFVQALGDFSLDGVEVRLGVGAVSRL
NRQFT)

20.
AAAACCGGGTACCAAACAGCAGTTCCCGTAATCGGTTCCGCATTCCCAGCTTCACCAGTAACCGGTAT
TTGTCCTGCCAGATTTGCTTCTCCTGTGTATTCAGCCTTCCACACAACGCTGACAACCTTCCTCGTCC
ACGGTAAACTTCCTGAAGCAAGCGTAACTGGGCCAGGCTCACTCCGCATGCCAGTATCTCCCG
(KTGYQTAVPVIGSAFPASPVTGICPARFASPVYSAFHTTLTTFLVHGKLPEASVT**GPGSLR**MPVSP)

21.
GGACTGACGCTAATGTCTTTGACGTCGTTACGCAGCAGGTTTGGATCGGTATAAGGAATGATCAGGCT
GATAATCAAAATCGCGAAAACATAAAACAGCAGAATACGCCAGAACACCTGACGCACCGCGCGTGGAA
TATTCTTCTCCGGGTTTTCAGATTCCCCGG
(TDANVFDVVTQQVWIGIRNDQAD**NQNREN**IKQQNTPEHLTHRAWNILLRVFRFP)

22. DELETION MUTANT

23.
CCTTCCGAGGTGAATGACAAGATCCGCGCGATCGCGATCCAGGCTTATCAGACGCTGGGATGCGCGGG
TATGGCGCGTGTCGATGTCTTCTTAACGGCAGACAATGAGGTCGTGATTAACGAGATCAATACGCTGC
CAGGTTTTACCAATATCAGTATGTATCCAAAACTCTGGCAGGCGAGCGGACTGGGCTATACCGATTTG
ATCAGCCGTCTGATTGAGCTGGCGCTGGAACGCCATACGGCGAATAACGCCTTAAAAACCACGATGTA
ATAGCGCACGCCCGATAGCGCAACGTTTATCGGGCGTCTTTTACCTCATCCTCTTCGCGCCGGCGAAT
AATGAACCCCGCCAGCCAGAAGCT
(NO ORF)

24.
CTCCGACGAACAGATCGAAAAGCTGGAAGAAGTCTTCCACGCCTCAATGTTCGACTATCAGCGTCACT
GGTTTGAAGCAGGAAAAACAAACCGCATCCGTAATCTGCTCAAGTCGCGCCAGATTGGCGCCACGTTT
TATTTTGCCCGTGAAGCATTGATTGACGCCCTGCTGACCGGACGCAACCAGATTTTCCTTTCTGCCAG
TAAGGCACAGGCGCACGTCTTTAAGCAGTACATCATCGACTTTGCCAAAGAAGTTGAGGTGGAGCTGA
AAGGCGATCCTATGGTGCTACCCAATGGCGCAGCATTGTACTTTCTCGGCACCAACGCCCGTACGGCG
CAGAGCTACCACGGCAACCTGTACCTTGATGAATATTTCTGGATACCGAAATTCCAGGAACTGCGCAA
GGTGGCCTCCGGTATGGCCATTCACAAGAAATGGCGACAAACCTACTTTT
(SDEQIEKLEEVFHASMFDYQRHWFEA**GKTNRI**RNLLKSRQIGATFYFAREALIDALLTGRNQIFLSA
SKAQAHVFKQYIIDFAKEVEVELKGDPMVLPNGAALYFLGTNARTAQSYHGNLYLDEYFWIPKFQELR
KVASGMAIHKKWRQTYF)

25.
GGATGTCAGTCGTACGGGAATGTTCTTCGAAGGTTTCGGCGTAGACCATGTGCACAGTAAATTAAGCC
CCATGCACGGTACTGGCGATTTAACCCACTGGAAACCGATTGAATCGCGGCAGAATAAATTTTTCGAA
CAGTATGAAGGATATCTGTCG
(DVSRTGMFFEGFGVDHVHSKLSPMHGTGDLTHWKP**IESRQN**KFFEQYEGYLS)
26.
GCGCGCCAAAGTCAAAAGTGGCGGCCACCTGTGATTATTCGGCGGAAGTGGTGCTCCACGGTGACAAC
TTCAATGACACCATCGCTAAGGTGAGTGAAATTGTCGAAACCGAAGGCCGCATTTTTATTCCGCCTTA
CGACGATCCAAAAGTCATTGCCGGACAGGGCACTATTGGTCTGGAAATTATGGAAGATCTGTACGATG
TCGATAATGTCATCGTGCCTATTGGCGGCGGCGGTTTAATTGCAGGTATTGCCATTGCGATTAAATCG
ATTAACCCGACCATTAAAGTTATCGGCGTACAGGCCGAAAATGTTCACGGTATGGCGGCGTCTTATTA
CACTGGCGAAATAACCACGCACCGAACGACCGGCACCCTGGCGGATGGTTGTGATGTCTCCCGCCCGG
GTAATTTAACCTATGAAATCGTTCGTGAATTAGTCGATGACATTGTCCTGGTTAGCGAAGATGAAATT
CGTAACAGCATGATCGCTTTAATTCAGCGCAATAAGGTTATTACTGAAGGCGCTGGCGCTCTGGCTTG
CGCCGCACTATTAAGTGGGAAATTAGATAGCCATATTCAGAAC
(APKSKVAATCDYSAEVVLHGDNFNDTIAKVSEIVETEGRIFIP**PYDDPK**VIAGQGTIGLEIMEDLYD
VDNVIVPIGGGGLIAGIAIAIKSINPTIKVIGVQAENVHGMAASYYTGEITTHRTTGTLADGCDVSRP
GNLTYEIVRELVDDIVLVSEDEIRNSMIALIQRNKVITEGAGALACAALLSGKLDSHIQN)

27.
ACAGAAATAACCACACCAGGAGTGCAACTTATTACCCGGCTAACGGTCTGAGGTGCAGTGATTATTGC
ATGAAAAAGGATTTTACCATGAAGAAAATTGTTTGCGCCGTCGTTGCGCTCCTGCTGACCCTACCCGC
CTGGGCG
(R**NNHTRS**ATYYPANGLRCSDYCMKKDFTMKKIVCAVVALLLTLPAWA)

28. SEQUENCE NOT OBTAINED

29.
CCTGGCGTTCATGCCCTCGTGGTTTAGTACTGACTTAGTTTTACACTCCTTACGGGAACCATTGTCGT
ACATGATGGCCTAACTATACGTAAATCATTCAAGTTACCCTGATGAGCTTACTCAAGTAAGTGCTTCG
GATAAGCGAACGCAACTTGAAGGATGATGCGTATTTCAGAGTGTACCGCTGCGGATAATGACTCTTCT
GCGATACATTATTTTCGTATCTACAGAAGGTATTTTATGTTTCCTCAAAGCAAATTTTCACGCGCGTT
TTTGCACCCGCGCTACTGGCTGACGTGGTTTGGCGTTGGCATCCTCTGGTTACTGGTGCAGCTTCCCT
ACCCGGTTTTGCGTTTTCTGGGCACCCGCACCGGTAAACTGGCGCGCCCCTTCCTGAAGCGACGTGAG
TCAATCGCCCAGAAAAATATTGAACTCTGTTTTCCGACCCTCTCCCGGGAAGAAAGGGAAAAACTGAT
CGCCGAGAATTTCCACTCTC
(NO ORF)

30. DELETION MUTANT

31. GCAACAAAGTTGAGACTAACCATGAGCCATAACGACACTATCGTCGCCCAGGCAACCCCACCG
(ATKLRLT**MSHNDT**IVAQATPP)

32.
CCCGAAAAAACCGGGAAACATCTTGGCCCATATAATGACAAAGGGCATTTGGGGGATCTGCCTGGACT
GGTTGTCAATGCAGATGGTACAGCCACGTATCCGTTACTGGCACCACGCCTTAAATCACTGTCAGAAC
TGAAAGGTCACTCATTGATGATCCATAAAGGCGGTGACAATTACTCCGATAAACCTGCTCCACTG
(PEKTGKHLGPYNDKGHLGDLPGLVVNADGTATYPLLAPRLKSLSELKGHSLMIHKG**GDNYSD**KP
APL)

33.
AAGCCGCCGGAGGCGATAGCGATTTTCCAGCCGAGCGCTTCCAGTTTTATCACCAACTGCGTTAATCC
TGGCATTAGCGGCAGATTCCCGCGCACCTGTCGCAAAATATCGGCATCCGCGCCTTTCAACGTCGCCA
CGCGGCTGCGCAGGCTGGCGGTAAAATCAAGCTCGCCGCGCATCGCGCGCTCCGTCACTTCAGCCACC
TTCTCACCGGTACCGGCCAGCTTC
(KPPEAIAIFQPSASSFITNCVNPGISGRFPRTCRKISASAPFNVATRLRRLAV**KSSSPR**IARSVTSA
TFSPVPASF)

25.
GGATGTCAGTCGTACGGGAATGTTCTTCGAAGGTTTCGGCGTAGACCATGTGCACAGTAAATTAAGCC
CCATGCACGGTACTGGCGATTTAACCCACTGGAAACCGATTGAATCGCGGCAGAATAAATTTTTCGAA
CAGTATGAAGGATATCTGTCG
(DVSRTGMFFEGFGVDHVHSKLSPMHGTGDLTHWKP**IESRQN**KFFEQYEGYLS)
26.
GCGCGCCAAAGTCAAAAGTGGCGGCCACCTGTGATTATTCGGCGGAAGTGGTGCTCCACGGTGACAAC
TTCAATGACACCATCGCTAAGGTGAGTGAAATTGTCGAAACCGAAGGCCGCATTTTTATTCCGCCTTA
CGACGATCCAAAAGTCATTGCCGGACAGGGCACTATTGGTCTGGAAATTATGGAAGATCTGTACGATG
TCGATAATGTCATCGTGCCTATTGGCGGCGGCGGTTTAATTGCAGGTATTGCCATTGCGATTAAATCG
ATTAACCCGACCATTAAAGTTATCGGCGTACAGGCCGAAAATGTTCACGGTATGGCGGCGTCTTATTA
CACTGGCGAAATAACCACGCACCGAACGACCGGCACCCTGGCGGATGGTTGTGATGTCTCCCGCCCGG
GTAATTTAACCTATGAAATCGTTCGTGAATTAGTCGATGACATTGTCCTGGTTAGCGAAGATGAAATT
CGTAACAGCATGATCGCTTTAATTCAGCGCAATAAGGTTATTACTGAAGGCGCTGGCGCTCTGGCTTG
CGCCGCACTATTAAGTGGGAAATTAGATAGCCATATTCAGAAC
(APKSKVAATCDYSAEVVLHGDNFNDTIAKVSEIVETEGRIFIP**PYDDPK**VIAGQGTIGLEIMEDLYD
VDNVIVPIGGGGLIAGIAIAIKSINPTIKVIGVQAENVHGMAASYYTGEITTHRTTGTLADGCDVSRP
GNLTYEIVRELVDDIVLVSEDEIRNSMIALIQRNKVITEGAGALACAALLSGKLDSHIQN)

27.
ACAGAAATAACCACACCAGGAGTGCAACTTATTACCCGGCTAACGGTCTGAGGTGCAGTGATTATTGC
ATGAAAAAGGATTTTACCATGAAGAAAATTGTTTGCGCCGTCGTTGCGCTCCTGCTGACCCTACCCGC
CTGGGCG
(R**NNHTRS**ATYYPANGLRCSDYCMKKDFTMKKIVCAVVALLLTLPAWA)

28. SEQUENCE NOT OBTAINED

29.
CCTGGCGTTCATGCCCTCGTGGTTTAGTACTGACTTAGTTTTACACTCCTTACGGGAACCATTGTCGT
ACATGATGGCCTAACTATACGTAAATCATTCAAGTTACCCTGATGAGCTTACTCAAGTAAGTGCTTCG
GATAAGCGAACGCAACTTGAAGGATGATGCGTATTTCAGAGTGTACCGCTGCGGATAATGACTCTTCT
GCGATACATTATTTTCGTATCTACAGAAGGTATTTTATGTTTCCTCAAAGCAAATTTTCACGCGCGTT
TTTGCACCCGCGCTACTGGCTGACGTGGTTTGGCGTTGGCATCCTCTGGTTACTGGTGCAGCTTCCCT
ACCCGGTTTTGCGTTTTCTGGGCACCCGCACCGGTAAACTGGCGCGCCCCTTCCTGAAGCGACGTGAG
TCAATCGCCCAGAAAAATATTGAACTCTGTTTTCCGACCCTCTCCCGGGAAGAAAGGGAAAAACTGAT
CGCCGAGAATTTCCACTCTC
(NO ORF)

30. DELETION MUTANT

31. GCAACAAAGTTGAGACTAACCATGAGCCATAACGACACTATCGTCGCCCAGGCAACCCCACCG
(ATKLRLT**MSHNDT**IVAQATPP)

32.
CCCGAAAAAACCGGGAAACATCTTGGCCCATATAATGACAAAGGGCATTTGGGGGATCTGCCTGGACT
GGTTGTCAATGCAGATGGTACAGCCACGTATCCGTTACTGGCACCACGCCTTAAATCACTGTCAGAAC
TGAAAGGTCACTCATTGATGATCCATAAAGGCGGTGACAATTACTCCGATAAACCTGCTCCACTG
(PEKTGKHLGPYNDKGHLGDLPGLVVNADGTATYPLLAPRLKSLSELKGHSLMIHKG**GDNYSD**KP
APL)

33.
AAGCCGCCGGAGGCGATAGCGATTTTCCAGCCGAGCGCTTCCAGTTTTATCACCAACTGCGTTAATCC
TGGCATTAGCGGCAGATTCCCGCGCACCTGTCGCAAAATATCGGCATCCGCGCCTTTCAACGTCGCCA
CGCGGCTGCGCAGGCTGGCGGTAAAATCAAGCTCGCCGCGCATCGCGCGCTCCGTCACTTCAGCCACC
TTCTCACCGGTACCGGCCAGCTTC
(KPPEAIAIFQPSASSFITNCVNPGISGRFPRTCRKISASAPFNVATRLRRLAV**KSSSPR**IARSVTSA
TFSPVPASF)

34.
GAATCGGACCAGCAATAGCTGACGAACATATCAGCCCGTAATCTTTTTGATTGCCATCACCTTCGCGG
GTGAGGGCGTTGTTGTTCGTTAATACACCTACTTTGAGCCGGTTCACACTTTTCAATGAAAATTGCAG
ATCAATTTCATGATGAGTTATGTAGACTGGCCGCCATTAATTTTGAGGCACACGTACTACATGGCTGA
ATTCGAAACCACTTTTGCAGATCTGGGGCTGAAGGCTCCTATCCTTGAAGCCCTTACCGATCTGGGTT
ACGAAAAACCATCTCCAATCCAGGCAGAGTGCATTCCGCATCTGCTGGGCGGTCGCGACGTGCTGGGC
ATGGCCCAGACCGGTAGCGGCAAAACCGCAGCG
(MMSYVDWPPLILRHTYYMAEFETTFADLGLKAPILEALTDL**GYEKPS**PIQAECIPHLLGGRDVLGMA
QTGSGKTAA)

35.
CGCCGGAGCAAATGATCGGTGAAATGAGCCAGGAGCACCAGACCGATTTCGCCGGTTTTAATATTAGC
TACGACAACTACCACTCAACGCACAGCGACGAGAATCGAGAGCTGTCCGAGCTGATTTATACGCGCCT
GAAAGAGAACGGTTTTATTAAGAACCGCACCATCTCTCAACTCTACGATCCGGAAAAAGGCATGTTCC
TGCCGGACCGATTTGTGAAAGGCACCTGCCCGAAATGTAAATCCGCAGACCAGTACGGCGATAACTGT
GAAGTGTGCGGCGCGACCTACAGCCCGACCGAACTTATCGAACCGAAATCCGTGGTGTCCGGCGCGAC
GCCGGTAATGCGTGACTCCGAGCACTTTTTCTTTGACCTGCCGTCATTCAGCGAAATGTTGCAGGCGT
GGACCCGCAGCGGCGCGCTGCAGGAGCAGGTGGCGAACAAAATGCAGGAGTGGTTTGAATCC
(PEQMIGEMSQEHQTDFAGFNISYDNYHSTHSDENRELSELIYTRLKENGFIKNRTISQLYDPEKGMF
LPDRFVKGTCPKC**KSADQY**GDNCEVCGATYSPTELIEPKSVVSGATPVMRDSEHFFFDLPSFSEMLQA
WTRSGALQEQVANKMQEWFES)

36.
GTAAATGTTTTTATATTCGTCCATATCGACGCTGATGATGATTTCCAGGTACCGGATTGGCACGCTTG
CAGAGAATGATTGTTGGCATTATTTTTTGCCTCTTTTGAGGTTAGCATGCCGTCTCCACGCATCCGTA
AAATGTCCCTGTCACGCGCACTGGATAAGTACCTGAAAACAGTTTCTGTTCACAAGAAAGGGCATCAA
CAGGAGTTTTACCGGAGCAATGTTATCAAGCGATATCCCATTGCCCTTCGGAATATGGACGAAATAAC
AACCGTTGATATTGCTACATACAGAGACGTTCGTTTAGCAGAAATCAACCCCCGAACGGGTAAACCCA
TTACAGGTAATACTGTACGTCTTGAACTCGCCCTTCTGTCATCTCTGTTCAATATTGCTCGTGTTGAA
TGGGGAACCTGTCGTACTAACCCGGTTGAACTGGTTCGCAAGCCGAAAGTATCCTCGGGACGAGATCG
CCGGCTAACGTCTTCAGAAGAACGTCGCCTTTCTCGCTATTTCCGCGAAAAAAATCTGATGTTGTATG
TCATTTTCCATCTTGCCCTTGAGACAGCCATGCGGCAGGGCGAAATACTGGCCTTACGTTGGGAGCAC
ATTGATTTGCGCCACGGTGTGGCTCATTTACCTGAAACCAAAAACGGTCACTCACGG
(MPSPRIRKMSLSRALDKYLKTVSVHKKGHQQEFYRSNVIKRYPIALRNMDEITTVDIATYRDVRLAE
INPRTGKPITGNTVRLELALLSSLFNIARVEWGTCRTNPVELVRKPKVSSGRDRRLTSSEERRLSRYF
REKNLMLYVIFHLALETAMRQGEILALRWEHIDLRHGVAHLP**ETKNGH**SR)

37.
GTGAAGGCGCGGAAAGGTCACTGGATAACGATGTCATTGCCTCTTTTGATAAGCCGTTCTCTCCTCAC
GGCGGTACTAAGGTGCTAAGCGGTAATCTGGGGCGCGCAGTAATGAAGACGTCTGCGGTACCGGTTGA
AAACCAGATCATTGAAGCGCCTGCCATGGTATTTGAAAGTCAGCATGATGTGCTGCCTGCGTTTGAC
(EGAERSLDNDVIA**SFDKPF**SPHGGTKVLSGNLGRAVMKTSAVPVENQIIEAPAMVFESQHDV
LPAFD)

38.
CGATTACAGAACATCACGTTTTTAACGTTTGCAGCACAATAAAAAACGGCTTCAAAGAAGCCATATCG
GCGTCTCGTAGGGGGAATAAGATGAATATTTTAGGTTTTTTCCAGCGGCTGGGT
(ITEHHVFNVCSTIKNGFKEAIS**ASRRGN**KMNILGFFQRLG)

39.
TTGATCTAAAATTTCATGATATTCCCAACACCACGGCCCGGGCGGTCGCCGCTGCGGCAGACCTGGGC
GTCTGGATGGTAAACGTTCACGCGTCTGGC
(DLKFH**DIPNTT**ARAVAAAADLGVWMVNVHASG)

40.
GATTCAAGATCGAAACGTTTTACACCTAAATACATTTTCGGTGACGAATGTGAAGGTAAACAGTCCTC
GTTCAATACTTCCATCGGCGAGGAAAGTGCAAAGAGTAAATATTGTGATTCTTTATCCATAATAAATC
GCCAAAATATATTCACACCACAAATAAAAACGCAATTGAAAACTAAAATAGAGTACAACAAAGCCAAA
TCGCAATATATTATTAATCTAAAAAACAATGGCGGGATAAATTAAAAAATGAATCATTCCATAACCAG
TCATCCCTGCGACAACGTATCTTTAGCACAATTAACCGAACTGGCGCAGTCAGGAAATAGTGAAGCTC
AATATATATTAGGCCGTTTATATAATGACGAACGTATAGATGGCAGCGAAGAGGATAAGCTCTCTTTT
TATTGGTTGCAGCAGGCAGCTGAACAAGGACACTGCGAGGCACAATATTGGCTCGGCTTACGATACAA
AGACACGCCTACCGACATGAAAGATAATACGCTGGCTTTATTC
(MNHSITSHPCDNVSLAQLTELAQSGNSEAQYILGRLYNDERIDGSEEDKLSFYWLQQAAEQGHCEAQ
YWLGLRYK**DTPTDM**KDNTLALF)

41.
GATCCTATGAAACAAAAAGTGGTTAACATTGGCGACATTAAGGTGGCAAATGACCTGCCGTTTGTGCT
GTTTGGCGGTATGAACGTGCTGGAGTCACGCGATCTGGCAATGCGCATTTGTGAGCACTACGTAACC
(DPMKQKVVNIGDIKVANDLPFVLFGGMNV**LESRDL**AMRICEHYVT)

42.
GCGATTACGATCAAACGCTCCTGGGCGGGCTGACGTCGCCTGTTAAAGGGCTGCAAAACGATCTGGGT
ATAAAACGCTATATGGCGGCACTCGGGAGCGACAGCTATATCGTGATGATCGACCCGGCGTCCTTTAT
CGATGTGATTCCGTTTGGCGCATGGCCGATTGACGTCGCTATTATTGGCCGGGAACGCAATACTGTGG
TTGCCAGCAGCGGTAACCTTGACCCCGCTATTCTTCCGCTCATTCACCATGAGACGCCCCTGCGGCTG
GAAAACCGAGGCATTCAGTATGCTATCCACCCCTTTCCGGAAATGGGCATTACTATCGTAAGCTGGGC
ACCGACCGCTCCGCTGGAAAGGAGCTGGTATCGACAGGCGTTTATCTGGCTACCGGCGGGCATCGTAA
CGGGTCTGCTGGCCGCGGCCTTTATTCTGCGTATTTTGCGTCGACTACAGTCGCCCCGCCACCGTCTC
CAGGATGCGATAGACAATCGCGAAATTAACGTACACTATCAGCCTATTGTCTCCCTGAGCAGCGGAAA
AATTGTCGGTGCCGAAGCCTTAGCTCG
(DYDQTLLGGLTSPVKGLQNDLGIKRYMAALGSDSYIVMIDPASFIDVIPFGAWPIDVAIIGRERNTV
VASSGNLDPAILPLIHHETPLRLENRGIQYAIHPFPEMGITIVSWAPTAPLERSWYRQAFIWLPAGIV
TGLLAAAFILRILRRL**QSPRHR**LQDAIDNREINVHYQPIVSLSSGKIVGAEALA)

43.
ATGATACCCGCGACCAGAGAATCGGTTTTTTTCAGGAACGGGACGAAGTTACCCAGCAAATCCTGCAA
CATAAAGCGGCCGGAACGGGTACCCGCATCCAGCGCGGTCAGGATAAACAGCGCTTCGAACAGAATAC
CGAAGTG
(MIPATRESVFFRNG**TKLPSK**SCNIKRPERVPASSAVRINSASNRIPK)

44.
AGCCTTCAAACGCCAGCTGAGCGCGGACGCTTTCTGCCTGCTCGGCGCCTTTGAATGAACCACATTGC
ACCATCCAGCG
(NO ORF)

45. DELETION MUTANT

46.
CGATAAGCGCGCCGACCAGCGTCGCCAGCACCATCACCGGAAGATTAGCGCACTTCATCACCAGCAGA
ATGCCAATTCCCAGAGAGCACAGACCAAAAATAGACGTCATTGAGACGCGAATCCGTTCTGGCAGACG
CTGACTTAACAGCGCGCCAATAACGCCGCCGAATAAAATTGCGCCAGCGTTAATAAAAGGTCCAATTA
CCACAATGACTCCTGTTATTTGTCTTTCAAATTGCCTTATTATCACGCGATTAAGCTGTTAATAGCTT
GATTATCTGTGACGTATAGAGCGCTGATTTTACCTTGCGCCGCCTGGTGAAAGGTGCCATGATTGCGC
GAAATTTCTCCTGCCAGCATGGGGCTCGCCGGGATGAACATAGCGCCTTTATATTGTCTTCACTGTCG
CCTGACTGCGCGAGGTTTGCCCCTTTCTTTCCTTTTTTTGTCATCCCCGCTATGCGGTGAGGGCAGCG
CACGCTCACTGCAGGACAACAGTAAAATCAGAAACAGTCTGTTTTTATTGATGTCTGGTGGTCGG
(NO ORF)

47.
CCCCGGGCAGGATCTACCCAGTCAGGCCGCTCAATATGTTTGAGATCGGATGGCGACATATCAAACTG
CCAACAGTCATTGCTGTTTTCAGCCTGAAATCTTACCGCCGGAGGCTCCCGTAACAGGC
(NO ORF)

48.
ACTAACATGAGAACCCATACGACGCCATGAGCGACGACAATTCACACAGTAGTGACACAGTAAACAGT
AAAAAGGGATTTTTTTCCCTGCTACTCAGCCAGCTTTTCCACGGTGAACCTAAAAACCGTGATGAGTT
GCTGGCGCTGATCCGTGATTCCGGGCAGAACGAGCTTATCGATGAAGATACGCGCGATATGCTC
(MS**DDNSHS**SDTVNSKKGFFSLLLSQLFHGEPKNRDELLALIRDSGQNELIDEDTRDML)

49.
AATCACGCCACAGCGCATGGGTCAGGCATTTATCGCCGCCCTGGCAGCCGCCTTTGCCCTGGCAACGG
GTCGCGTCAACGGATTCATCAACCGCGCTGATCACTTCGCCGACCGCGATACTGCCAGCATCTTTACC
CAGCAGATAACCGCCGCCCGGCCCGCGTACGCTGGAAACCAGACCGTTTTTACGTAAACGGGGACCAC
AAAAAATTTATTTCTTTGTTAATGCACATGCTGCGCAGCCGCATCAGTGACAGCGTTTACCACATCAT
TCAGAGCGATATTCATAAAGACTGGAATCTG
(NO ORF)

50.
GGTATGGTGATGGATTTGACGCCAACGGTGTTAATTCTTACCCCTGTATTATTGCCATTAGTTAAAGA
AGCCAATATTGACCCGATTTATTTCGGCGTCATGTTCATTATTAACTGCTCTATTGGATTAATCACAC
CGCCCGTTGGCAACGTCCTCAACGTTATTTCCGGGGTAGCAAAATTGAAATTTGATGACGCGGTAAGA
GGCGTATTCCCTTACGTTGTCGTCCTGATGTCGCTGCTGGTTTTATTTATTTTTATTCCCGAGCTAAT
TATCACACCGCTTAAATGGATTAATTAAAGGATAATATTATGAAATTACACGTTATTGCTCGTTCATT
ATTGATAGCTGGTCTGACGGTTTTCAGCGTGTCGTCTCTGGCGGCGCAATCTTTACGTTTTGGTTATG
AAACACCGCAAACTGACTCCCAACATATTGCCGCGAAAAAATTTAACGAACTATTAAAAGAAAAAACT
AACGGCGAATTAACGCTAAAACTCTTTCCT
(NO ORF)
51.
AAAATAAAAACTCATGCACAAACGACCACGTTATATACGGTGGCGGACATCTTATTTTATTGGCTTGC
TGAATTAAAACGAGAATATCAATATGAGTAATGAGACGGGACATCTGAACAGACGTTCTTTTTTAAAG
GGGATAGTGGCACTGGGCGCCGTTGCGGCTTTACCCGGAGGATTACTGACCTCCCGATGCGCGCTTGC
G
(NO ORF)

52.
CCTGTGAATGGTTACACGTCTTCATATTACTTTGCATTCGCTTTCCGCGTGTGCAGCGTGACGGGTTA
GGCTATAATCCCCCCTTTTACAACAGACTAAAAAACCTCAACTTTGACCATTACGAAACTTGCATGGC
GTGATCTGGTTCCGGATAGCGAAAGCTATCAGGAGATATTTGCACAGCCACACGCGACTGACGAAAAC
GACACCTTACTCAGTGATACTCAGCCACGACTGCAATTTGCGCTTGAGCAACTTATACAGCCGTGGGC
ATCATCCTCTTTTATGCTGACTAAAGCGCCTGAAGAGCAAGAGTATCTCACTTTACTTTCAGATGCCG
TCCGCGCTCTGCAAACCGATGCCGGACAATTAACCGGCGGACATTATGAC
(LTITKLAWRDL**VPDSES**YQEIFAQPHATDENDTLLSDTQPRLQFALEQLIQPWASSSFMLTKAPEEQ
EYLTLLSDAVRALQTDAGQLTGGHYD)

53.
CGTCCACGCGGATAAGCCAAACGTCCCACGCGGCCTGACAAAATATCTCACCGCGCTGCATGAAGAGC
TTTCCAGCCATATGATGAAAGAAGAACAGATCCTGTTCCCCATGATCAAACAGGGTATG
(VH**ADKPNV**PRGLTKYLTALHEELSSHMMKEEQILFPMIKQGM)

54. CCTGTGAGAAGCTCAGTTCCTCGTCGCCCTCATGC
(**HEGDEE**LSFSQ)

55.
AGCCAGACACCTCCGATAAGAAGATACAGCCCACAAAGTGCTGCAAAGAGGGCCGTCAGCGTCACGAG
TAATCGTGGCGATCGTGCGTTATTTTCTGCCATAAAAAGACACCATCCCAAATTGTTAATTTTTTAGT
AGCAATTAAGTATAGGAATTAACATGTGTGATCGTCATCACAAAATGAGCTTTCTTATCAAATGCCGC
GAATGAATACGTTTGCTGTTATACTGCGTGCCTTGCGCGTCAATGCGGCGTTATTAGTCACCGGCATT
GAGTGATTTGTTTTTAAATCATATGGTTATAAATATGAAACATACTGTTGAAGTCATGATCCCCGAAG
CGGAGATCAAAGCACGTATTGCCGAGTTGGGTCGTCAGATTACCGAACGTTATAAAGACAGCGGC
(NO ORF)

56.
ACGTAGTACGTGAGGTTTGACTCGCTACGCTCNCCCTTCGGGCCGCCGCTAGCGGCGTTCAAAACGCT
AACGCGTTTTGGCGAGCACTGCCCAGGTCCAAAATGGCAAGTAAAATAGCCCTACTGGGATAGGCTCT
AAGATAATTGCTCGCCATTCGTGGGTAATAAAAACGGNTAATCACTCCCTTATCAGGATATCTTCATG
AAAAACTTTGTACGCACAACTTTACTGGCAGCGACTCTGGCGGGCGTTTCTTTTGGCGCTTTCCC
(NO ORF)

57.
GCGTGAGTCCGGCCCGGGCAATAAAAGGTTTTAGCGCGCTGAATACGGGGTCAAAGGAGGCGGGATAC
AGCGGGCAGCCGCAAAGATCGACCGGCGTACCGTCGCTATACAGCATCCCGAAGAGCGGTTTTTCTAC
AATACC
(VSPARAIKGFSALN**TGSKEA**GYSGQPQRSTGVPSLYSIPKSGFSTI)

58.
CAGCGCTCATTAATATATTGTATTTATTTTAACATGTTAAGTGAAAAATTCAATTGAGTAATCACCAC
GAATCACTGGAAGGCTTTCTTCGTAAACGGCATAGTGTAAGTAAGCTGGTTGTGCATTAATCTTTACG
ACAAAATACCGCTGCAAGCTGTTTGACGGGCAGATTATCGCTCAGTTACGTGATGCTTTTGGTTCGGC
TGCGGCAAAGTTGGAGTGCGAAATTATCGAGATGGATGGTGAACAAGATCACGTCCATCTGCTGATAG
CGTATCCGCTAAAACTGGGGGTCAGCGTGATGGTAAATAATTTAAAGTCGGTATCGTC
(NO ORF)

59.
CAATCTTTGTGGTTAACTTTTAATGATACAGCGTCAGGTGTGCTACCTGAAATAGCAGTAAGTTGAGT
ATACGCTGAGCCAGAACCGGCCCCCCCGTTAATGTGGGAGTACAATGCTCCGCTATCTAATGTCTTTG
ATAACTTATGAATCC
(NO ORF)

60.
GCAGTTGCTGGCGAATATTGTCCAGTTGCTTACGCCCGTCTGGTTGCACGCTGTAGCGGCCTTCAAAT
CCCAACCCCAGTAGGTGGTAGATAACCTCCAGTACGTTGCCAATGCTCCTGGAAGCTGGCCGCCAGAC
GCCCGATGATCTGGAAAAGTTTTATCCCGCCTTCGCTTTCACCATG
(SCWRILSSCLRPSGCTLWRPSNPNPSRWWITSSTLPMLLEAGR**QTPDDL**EKFYPAFAFTM)

61.
GACTAATTGTAATGAACTTAACAAATGGCTGTACCAGTACCCTTCTTTTTGAGCGATGATGGTGGCGC
TAACATAGTTCTCTTTTCTTTTTAAAGGGTTCATGTTTACTGTCGCCACCCTTACGCTAACTCCTTTA
GTTTCCTCCTTTGCTGTCATACTATCGAATGACGTGCCTCACCTGTGAACGCTAAGGATAATAATATG
GAAAATAAAGGCACAAATCTAACGCCCGAGCAGGCTCTGGCTCGC
(NO ORF)

62.
AAACCCCCGTTCGCCAGCGGCGGATACGGCGCTTCGAAATCGATTTTGTCGTACGGGTTGACCTTCTT
CTCCACGTCGAGGTGGAAGCTGTTGGTGTAGTAACCTTTATCGGTTACGCCCGGCACCACTCC
(TPV**RQRRIR**RFEIDFVVRVDLLLHVEVEAVGVVTFIGYARHHS)

63.
ATGATCAAGGCATCCGGCCTCATCAGGCGCTGGATCGTAAAAATATTCGCGAAGCGCTACACGACAGT
TTGACCCGATTGCAGACCGACTATCTGGATTTGTATCAGGTGCACTGGCCGCAGCGCCCCACTAACTG
CTTCGGTAAACTCGGCTATAACTGGACGGACTCCACGCCAGTAGTAAGTCTGCTGGAGACGTTAGACG
CGCTAAGTGAGTTTCAGCGAGCGGGTAAGATTCGTTATATTGGCGTTTCAAATGAGACAGCGTTTGGC
GTTATGCGCTATCTGCATCTGGCGGAAAAACATGACCTGCCGCGTATTGTCACGATCCAGAACCCCTA
TAGCCTGTTAAACCGCAGCTACGAGGTCGGGCTGGCGGAAGTCAGCCAGTATGAAGGTGTTGAATTGC
TCGCCTACTCCTGTCTGGCGTTTGGCACATTAA
(**DQGIRP**HQALDRKNIREALHDSLTRLQTDYLDLYQVHWPQRPTNCFGKLGYNWTDSTPVVSLLETLD
ALSEFQRAGKIRYIGVSNETAFGVMRYLHLAEKHDLPRIVTIQNPYSLLNRSYEVGLAEVSQYEGVEL
LAYSCLAFGTL)

64.
CAGAACTGCTGGGTAAACGCCGTCTGGAAAAATTCGCCGCGAAAGTTCAGCAGCAACTGGAAAGCAGC
GATCTGGATCAGTACCGTGCGCTGCTGGCGAAAATCCAGCCGTCTGCCGAAGGTGAAGAGCTGGATCT
CGAAACGCTGGCCGCCGCACTGCTGAAAATGGCGCAGGGCGAGCGTCCGCTGATCCTGCCGCCGGATG
CGCCGATGCGTCCTAAGCGTGAATTCCGT
(ELLGKRRLEKFAAKVQQQL**ESSDLD**QYRALLAKIQPSAEGEELDLETLAAALLKMAQGERPLILPPD
APMRPKREFR)

65.
GATGCNCTGCTGAAATGACAAAAGCTGCTTATGCGCTTCCGTCATTTTTTCGGTCGTGGAAAGCTTCG
CGGCGGCAATCAGCCCTTCAGTCTGCGCCTGTTGCTGGCTGTACTGCAAAAGCAGACGCCCGGCCTCG
TCGTTGTAGTAAGCCTTTGGCTTTTCCTTCTGCCGTGCCATTGCTTTTTTATGGCGTTCGTTTTCACG
CTCCAGCGCGGCATTGCGTACCGCTGCATCGGCATACTGCATGGCGGTAATGCGCGCCACCTCCCGTT
GATGCCGCAGGGATTCAGTTTCATTATCCCGG
(NO ORF)

66.
GCCATTAACTCGCTGACGCTCTCCCCTGCGCTGGCTGCCATTTTGCTAAGACCGCACCACGATACTGC
GAAGGCTGACTGGCTAACGCGGTTGATGGGCACGGTCACTGGCGGTTTTTTCCATCGCTTTAACCGTT
TCTTCGACAGCGCGTCGAACCGCTATGTTAGCGCCGTCCGTCGGGCCGTGCGCGGCAGCGTCATTGTG
ATGGTGCTCTATGCTGGCTTTGTGGGGCTGACCTGGCTTGGCTTCCATCAGGTGCCGAACGGGTTTGT
GCCTGCGCAGGATAAATACTATCTCGTCGGCATCGCCCAGCTCCCAAGCGGCGCATCGTTGGATCGCA
CAGAGGCGGTCGTGAAACAGATGTCCGCTATCGCGCTGGCGGAACCCGGCGTTGAAAGCGTCGTCGTC
TTCCCCGGTCTGTCGGTTAACGGCCCGGTAAATGTGCCAAATTCGGCGCTGATGTTCGCCATGCTGAA
ACCCTTTGACGAGCGTGAAGATCCTTCGCTTTCCGCTAACGCTATCGCCGGAAAGCTAATGCACAAAT
TTAGCCACATTCCCGACGGATTTATTGGCATCTTCCCGCCACCGCCGGTTCCAGGGCTTGGCGCGACG
GGCGGCTTTAAATTGCAGATTGAAGATCGTGCGGAACTGGGATTTGAAGCGATGACAAAGGTGCAAAG
CGAGATT
(AINSLTLSPALAAILLRPHHDTAKADWLTRLMGTVTGGFFHRFNRF**FDSASN**RYVSAVRRAVRGSVI
VMVLYAGFVGLTWLGFHQVPNGFVPAQDKYYLVGIAQLPSGASLDRTEAVVKQMSAIALAEPGVESVV
VFPGLSVNGPVNVPNSALMFAMLKPFDEREDPSLSANAIAGKLMHKFSHIPDGFIGIFPPPPVPGLGA
TGGFKLQIEDRAELGFEAMTKVQSEI)

67.
CCGTTTCCAGGGCATTGATTCAGGCTATTCGCACTGCTATTTTAGCCAGAGAGACAACCTCGCTGTAT
CGGCGAGGTTGTGTTAGCATTACCCTTCTATGAATCCACCTTCCAGCGTTTCAGAGCCTGCCAATGAG
TTATCAGGTCTTAGCCAGAAAATGGCGCCCACAAACCTTTGCTGACGTCGTCGGCCAGGAACATGTGC
TG
(MSYQVLA**RKWRPQ**TFADVVGQEHVL)

68.
TCGCATAACTACATGATTGCGATTCAAAAAACCGTTAAACCGATGTGGGAAGTGCGCAGCACGTATGA
CATCTGCGCGGATATCGCCGGGCATCTTGGACTGCGCGAGCAATATACCGAGGGCCGAACTCAGGCGC
AGTGGGCGGAGATGCACTATCAGCAGATCCGTGAAAAACGTCCATACCTACCGGAGTGGAGCGTGGCG
AAAGAGATGGGTGTGATTGACC
(SHNYMIAIQKTVKPMWEVRSTYDICADIAGHLGLRE**QYTEGR**TQAQWAEMHYQQIREKRPYLPEWSV
AKEMGVID)

69.
CAGGTTGTAAATGAGGTCTCTAAACTAAATCTGGTGCTGGATACCATTGATACAGCGATAAATAAACT
GGGCGAGCTAGTTGTTGATGATTTGCTGCAGTATCGAAACGTAAACCGCGTATATATTGTCGGTGGCG
GGGCTGCACTCATTGCTGATGCTGTTCGTAAGGCATGGAACCACCTTGGCGAAAAGGTGGTTCTTATG
GATGAACCTCAGACAGCGCTGGTCCAGGCTATTGCACGCTTCAAAGCGGAGGAGTAATCGATGTACGA
AGGCGAAGAAAGGAAAAAACTTTCACTCTACCTTCACCCGGAGGACTCTGCCGATTGTCTTGCACTGG
CTGAAATCGAAACTGTTCCCCGG
(NO ORF)

70.
GGTAAATTTCGCCTAATCCCAGCGAGTAAAAATCGACAACCGCCTGATCGGGATCGAGACCATCCGTT
TTATTCGCCACCAGGAAGGTCGGTTTTTCACGGGAGCGCAGATGTTTGGCAATCGCTTCATCCGCCGG
CATCAGACCAGCGCGCGCATCTACCATGAATAACACCACATCCGCTTCTTCAATCGCCAGCAGCGACT
GTTCCGCCATCCGCGTTTCGACGCCGTCTTCGGTGCCGTCAATACCGCCGGTGTCGATACAGATAAAC
TCGCGGCCTTCAACCTCCGCACGACCGTACTTACGGTCACGAGTCAGACCCGGGAAATCCGCAACCAG
CGCATCTCGGGTGCGGGTCAGACGGTTAAATAGCGTGGATTTTCCGACGTTAGGGCGCCCGACTAGTG
CGACCACAGGTACCATGTTTAAAGCCTCATTTAAAAAAT
(NO ORF)

71.
CGACTGCCAGGCAAACCATTAGTGGATATTAATGGTAAGCCCATGATTGTTCATGTCCTGGAGCGGGC
GCGCGAATCTGGCGCGGAACGCATTATTGTCGCGACCGATCATGAAGACGTGGCTCGTGCGGTAGAAG
CGGCTGGCGGCGAAGTGTGCATGACGCGCGCCGATCACCAGTCCGGCACCGAACGGTTGGCGGAAGTG
GTAGAGAAGTGCGGATTCAGCGACGATACCGTTATTGTGAACGTTCAGGGCGATGAGCCGATGATCCC
GGCGGTTATTATTCGCCAGGTCGCGGAAAATCTGGCGCAGCGCCAGGTTGGCATGGCGACGCTGGCGG
TGCCGATTCACAGCGCCGAAGAAGCGTTTAACCCGAATGCGGTAAAAGTCGTGCTGGATGCTGAAGGC
TATGCGCTTTACTTTTCCCGGGCGACGATTCCCTGGGATCGCGATCGCTTTGCAAAAAGTCTGGAAAC
GGTAGGG
(RLPGKPLVDINGKPMIVHVLERARESGAERIIVATDHEDVARAVEAAGGEVCMTRA**DHQSGT**ERLAE
VVEKCGFSDDTVIVNVQGDEPMIPAVIIRQVAENLAQRQVGMATLAVPIHSAEEAFNPNAVKVVLDAE
GYALYFSRATIPWDRDRFAKSLETVG)

72.
AATTTCTTAATGCAGTAGCCGCCTAATGGACCGGCGATCATCGAGCCGAGGAACATCGGCATATCCGC
GCCGACAATAACGCCCATGGTAGTGATAGCGCCCACTACGCCGCCGCGTTCGCCGCCAACCAGACGAC
CGCCGGTATAACCGATGAGCA
(NO ORF)

73.
ATCCTCTCGACGTCCTTATGAATCAATGCTTCTGCCGCCTTTGCCGCACTGATCCATGTTTTCCCGCA
GCCGGCTTCGCCGGTCGCAAAAATAAGCTGTTTACTGTCGATAGCGTTCAGATAATGCGCCTGTGCTT
CATTTCGCGCCTTAACAGGAGAGGTATCGCGACTATCGCGCGCCATGCCAATGGCTTCTACTCCGCCC
ATCTGTACCAGTGACGTGACCGATTCTTCTTCACGTTGCTTATGACTACGCGAATCTCGTCTCAACAC
ACGCTTTGCTTCACGACGAGCTTTGATCACTGCTTTTTGTCTTCCCATGGATAGCACCTTGAGTTGTA
GGTATACATCACCCGCGTTCTCTTGAACGCGATTATGCGCACACACATCTGAGGGTTGGCTTCCTTGT
AAGCCTTAGCTTGCTACTTGAAACGACACACACACGGCTACGCGCA
(NO ORF)

74.
CCGACGACAGCCATACCAACGGCAATAAAGACCGAGATGATGGCAAAACCCGCCAGGAATTTGACGTT
CTTGGTCATCATGTACATCACGGCACCGACCAGCACTTCCGCGAGAATAACCCACTTGACGGCGCTGG
AATCCTTACCAAATGTCGCCTTGACCGTACCGTTACCGCTGGCCATCAGGTCCTGCGAACCGCCGGCA
GCCATTGCCAGTTGCGGGAAAAAGAATATCAGTACTGCCGCCGGGATCACTGCGCGGGCAAGTTTCAG
CAGGTTCAGACGTGTGAACGTAAACAGAAACGATTTCTTTTTAACGGGCGCAGAACCACCCTGAACAC
TAAAAATAGCGCTCATATGGTTCCTCTTTTGAGTAATATCAACTATGAATATGAAAGTTAAATCTCTT
TATATGT
(NO ORF)

75.
GGCGCGACCAGCAGATCGCCCGGCTTGAGGAAAACGGTGGTGACCAGATGAATCGCCGACATGCCGGT
ATTCGTCAATACCGCGCC
(RDQQIARLE**ENGGDQ**MNRRHAGIRQYRA)

76.
GAAATAACCAGCCAGGGGTACTATATCTATGAAGCGCCGGTCCGTTTATGGCACTGGATCACGGCGTT
ATCCATTGTCGTCCTGGCTGTTACAGGATATTTCATTGGCCGCCCCCTGCCATCGATTCAAGGTGAAG
CGACCTTTATGTTTTGGATGGGCTGGATACGACTGATCCATTTTACCACGGCGTATATTTTTACTGTC
GCGCTGCTGTTTCGTATTTACTGGGCATGTGTCGGCAATGAGTACGCCAGGGAGATGTTTCTGGTTCC
GTTCTGGCGCCGCGCCTGGCGCAAAGGCGTTATCAGCGAAATCCGCTGGTATTTTTTCCTCGAAAAAG
AGGCCCATCGTTACTATGGACATAATCCGGTAGCGGGACTGGCCGTCTGGTTTGGTGCTGCTTTCCTT
GTAGACGAACACCCATTTGTGATGATTGCCGATTTGCTTTTTCAGCACCCGGCAAGCGGTATCATTCA
GCGCCACTCCAATGGCATGATTAGACTTGCTTTGTTCCG
(EITSQGYYIYEAPVRLWHWITALSIVVLAVTGYFIGRPLPSIQGEATFMFWMGWIRLIHFTTAYIFT
VALLFRIYWACVGNEYAREMFLVPFWR**RAWRKG**VISEIRWYFFLEKEAHRYYGHNPVAGLAVWFGAAF
LVDEHPFVMIADLLFQHPASGIIQRHSNGMIRLALF)

77. ACGCTGCTGGTAGACGCGCTGAAACGTCAGCACGGCGCGTCGTACC
(TLLVDAL**KRQHGA**SY)

78.
CGCGTAACTTAGACAATCTGCGACATCATCGCTTTTCGCCGACTTGCGGTTATCCCATGCCATTTCGA
TATCGAACACCAGTTTTTGCTCGATAGTCTGTTCCCAGTCGTAAACACCGATAGTGGTGATTACC
(RVTWTICDIIAFRRLAVIPCHFDIEHQFLLDSLFPVV**NTDSGD**Y)

79.
GCCAGGCTGTCGGTAAACGGCGTGGCGTTGAGCGCGCTGTAGGCCTCAATCGCATGCGTCAACGCATC
GATACCGGTCATCGCCGTCACGTTTGGCGGAACGCCTTC
(QAVGKRRGVERAVGLNRMRQ**RIDTGH**RRHVWRNAF)

80.
TGCTTCCCCATCCAGCGGTTAATTCTGTGCCTGGGTTATCGCGCGTCGTAATGATATTCACTACGCCG
CCGATAGCATCGGAACCATAAATAGCGGAGCGCGGACCGCGAATATATTCAATGCGCTGTACCAGCGA
CACCGGGAACTGGCTGAGATCGGCGGACCCGCTCACGCCTGCTAAATTCAGACGCACGCCGTCAATCA
ATACCAGTACATGGCTGGAGTTTGGTGCCGCAAATGAAAATGG
(NO ORF)

81.
ATATTCAGACAACAATTCCACGACTCCCACCTTGATTAAATAACGCGACTTGCGCTCAGTGTACAACA
GGCATCGAAGTCAGTCTCTCTTCATCCGTATGCCTGTTGAGGAAGAAAAAGTATGACAAGACGTTCAT
TTTACGGCGTTATCGCCCCTGTCTGATTGAAAAAAAAGGCGCTTTCAGGCATAAACCCGAAAAGTTAA
CGCGAAGATAAGACAACCTGGCAGGACGGTGCACCACACCGTTGAGGAGTAGCCATGCATTACC
(NO ORF)

82.
CTGCTGGCCGAGCGGGAAACCCTCAATGGCCTGAGTATGCTTGATCCATTAACCGGCTTATATAACCG
CCGTGGCCTGCAAAATCGGCTGGATACGTTACAGGCGCCGGGCAGCCATGAACACTACGTCCTGCTAT
TGGATATCGATCATTTCAAAGCGTATAACGATCACTACGGTCATATGATGGGCGATCAGGCGTTAATT
CGCGTCTCAGCGGCAATTCGGGACGCCGTGCGATCGCGCGATGTCGTCTGCCGTTTTGGCGGGGAAGA
ATTTCTGGTGTTGCTGACCACCGCCGAACCACAGCAAGCCCGCGCTACCGCAGAACGTATCCGACAGG
AAGTATACGATCTTAAAATCCCGCATATG
(LLAERETLNGLSMLDPLTGLYNRRGLQNRLDTLQAPGSHEHYVLLLDIDHFKAYNDHYGHMMGDQAL
IRVSAAIRDA**VRSRDV**VCRFGGEEFLVLLTTAEPQQARATAERIRQEVYDLKIPHM)

83.
CCGTGAATGACCGGTGTATTGTCGTCAGTGGTTGCGCCGCTGACAATAGTATCCTGGACTTCGCCGAC
GTTATCTTCGGCAAAAGTGATAACCGGATTGGCGGGC
(VNDRCIVVSGCAADNSILDFADVIFG**KSDNRI**GG)

84.
TGCCACTCGCTATAGCCTCCCGCCGGCGCAATGATTCCGACCGATGTACCGTGGGTGGCATAGGGAGG
CATGACCATGCCACTACAGCCACCGAGCAGTAACGCGGTGCCGACGATAAAAATGAACTTACCCAGAG
CGGTGTTTTTCATTAGTTCCTACCTATTTCTTCTCGTTGGTTATTTCTATGCCCTGCCTTGGCAGGGC
TCGCTATTATTCATTCTTCAACTGTGAGATCAAGTTATTTTAATTAAATCGGTATTATTCTTAATTGT
GTTCTTTATGAATAAATATCCTCCGGCTATGCCGGAGGATATTTATTATTTCCTCTCATAACTGAGAG
GCCCCACACAACCAGAGGGGGATGAATGTCCGAATCGATTTC
(NO ORF)

85. DELETION MUTANT

86.
CAGCGCAATTCATTTCCTTCTATTAACGAGAATAAATATTTGAATGCGATACTGAGGAAAGATAAATG
CAACAGGTAAAAATTTACACC
(NO ORF)

87.
ATCCATTTAAGGAGAAACTATGTCACGTATTCGTGTCGTAGCGGCCCGTCTGTACGCCGCATTTATCC
ACGCACAACTGTTTGTAATGTCATGCCGGAGCAAACTGACTAAGTTCCTGCTGCTCATTCCCATGATG
TTGCTGCCCAAAGCCGTTCTTGCC
(MSRIRVVAARLYAAFIHAQLFVMS**CRSKLT**KFLLLIPMMLLPKAVLA)

88.
TTCTGTCTAGCCAGNAAAGGAAAACNTCATTTTTTANAACCNGAAGCAGCGATTCAGAGAGGCAACAA
GTGGGGAACTGCTGAAGACCTAACTGCTGCCGAGTGGATGTTTGACNTGATAAAAAC
(FCLAXKGKXHFLXXEAAIQ**RGNKWG**TAEDLTAAEWMFDXIK)

89.
CAGGCTTTCATGAGCCCGTTTTTGCGCCGCATGATAAAGAAACCAATACTCAGGCGATGATCGCGACC
ATCGCCAGCGGCAAGGTGCATATAATTAGTCACCCCGGAAATCCAAAGTATCCAGTGGAGGTTAAAGC
CATCGCGCAGGCGGCGGCGAAACACCATGTAGCGCTGGAAATCAACAACTCTTCTTTTCTGCATTCGC
GTAAAGGAAGCGAAGATAATTGCCGCGCGGTCGCTGCC
(GFHEPVFAPHDKETNTQAMIATIASGKVHIISHPGNPKYPVEVKAIAQAAAKHHVALEINNSSFLH**S**
RKGSEDNCRAVAA)

90.
CGGCGGTGTTGATGGTGGCTGCCTGTGTCGGCGGCTATCTGTTGACGCCGAAATGGCAGGCTGTGCGT
AGCGAGCAACAGCGTCTGGCCGATCCGCTACGTGACTTTACGAACCCGCAAACGCCAGAGGCGCAGCT
TTCCAGG
(AVLMVAACVGGYLLTPKWQAVRSEQQRLA**DPLRDF**TNPQTPEAQLSR)

91.
CGCCGGTATTACTGGTTAACCATGCGCTACGTCCATTATTGTCCCGCTTTTTGCGGCGCAGTTTACCG
CAGCTGGTGGTGTTGTCGAACCTTGAGCTTTCCGATAACCGTCATATCCGCATGACGGCGACCATTGG
AGGAAAATAATGCGTAAATGGCTGGCGTTGTTGCTC
(NO ORF)

92. (SEQUENCE NOT AVAILABLE)

93.
GGCGCGCCGCAGTTTGATACGCAGGCGAACGTGATGCGTAAGTGCGACGGTTGTCTTGACCGACTGGA
GAACAATCTGCGCCCTATTTGTGTGGATTCTTGCCCGCAGCGAGCGCTGGATTTCGGCCCTGTCGATG
AATTACGGGCAAAATATGGCACGGAGAATCAAATCGCGCCGCTACCTTCGGCGTCGTTCACCCATCCT
AACCTCATTATTAAACCGCATCCGAAAGCGAGACCCACGGGCGATACGGAAGGCGCAATCATGAACAT
TCGGGAGGTG
(GAPQFDTQANVMRKCDGCLDRLENNLRPICVDSCPQRALDFGPVDELRAKYGTENQIAPLPSASFTH
PNLIIKPH**PKARPT**GDTEGAIMNIREV)

94.
GAACCGTCGCAAGTGGCAGGGCATTATCAAAGCGGTGGACGGTGAAATGATTACCGTCACAGTCGAAG
GCAAAGATGAAGTGTTCGCGCTGAGTAATATCCAGAAGGCGAACCTGGTTCCCCACTTTTAACAGTCT
GGATGAGGTGAAAAGCCCGCGATGAACAAAGAAATTTTG
(NO ORF)

95.
CGGCTGCTGAAGAGAATCACCCCGAGGCCGCCATCGCTGCCCATCATCGACATGGCTACCGAGCCTAC
AGCCTTCGCCTCCGGCAAGACCACCAGCAGTTCCGGGCCGTTTAGCGGGCCAAGATAATGCTGCGACT
GTCCCAGCC
(AAEENHPEAAIAAH**HRHGYR**AYSLRLRQDHQQFRAVWRAKIMLRLSQ)

96.
ATCTGCACTGGATTAATGTCGATGACGGGCGTTTTGTGGCCCAACAAAAAGTAGACAGTTCCGGTTTT
CTGACCGAACCGACGGTGGCGGATGGTAAACTGCTCATCCAGGCCAAAGACGGTACGGTCTACGCGAT
TACGCGTTAATCGTCACGGTTGTGTGTTTTAAAAAACGGCTCCTGGTGTAGGGGCCGTTTTCCTGTTT
TTAACAACGGCGTAAAAATGACGCGCGTTGTCTGATGATTTTTTAAATGAGGCTTTAAACATGGTACC
TGTGGTCGCGCTTGTCGGGCGCCCTAACGTCGGAAAATCCACGCTATTTAACCGTCTGACCCGCACCC
GAGATGCGCTGGTTGCGGATTTCCCGGGTCTGACTCGTGAC
(NO ORF)

97.
CAGGCGCTGGCCCGCCGCCGGCAGCTGCTGCGTCTCGCGCTTGCCGTAGAGGATTTCGCCGGGGGTAT
AGAAC
(**RRWPAA**GSCCVSRLPWRISPGVWN)

98. AACCCCGGCAAACCTACCGGCATAGTAC
(**TPANLP**AWY)

99.
GGCATATCCGGTCTGATTGCCAGCGCTTGTGAAAAATCATTTCGCGCTAATGCCCTCAAACCAAGACT
ATCATACAACACTCCGCGCTCATATAAAAGCTGTGCGCGTTCGTCATCGGTTAAAGCCCGACTGGCAA
GAATCTGTTCCATACGCGCCAGAATCACTTCCTGCTGTAAAGTCGGTTGCAATGGTACTGCGAGGACT
TCACTTTTACGCCAGGCGGAATTACTGCATCCTGCAAGCGTAAGTGCTGTCGCAACGAAACACCAGCG
CAAAAAAGGCTTCATTTCCCACTCCCGAAGACAACGATTGAATGAACGTCCTGTTCCCCGGTGGCTCA
ACAAGGCGTCCAGCCAGGTCTAAAGCCCTCCGCACTGCGGAGGGCAAAAGGCTACCTTACTCGGCCTG
TTCGCTCGCCGGAGCTTCC
(NO ORF)

100.
ACACTGATCGTCTTTACAGATTCCAGGTCAATGGTCGCCCTGACTTAAACAAAATGCACACGGCCATT
GATATGGGATCAAATAACCTGAATAACGTTGGGGCAGTAAATGCCCAGACAGGTAATTTC
(TDRLYRFQVN**GRPDLN**KMHTAIDMGSNNLNNVGAVNAQTGNF)

101.
CTTCATATAAACCACTGATATATCCATTTAAGGAGAAACTATGTCACGTATTCGTGTCGTAGCGGCCC
GTCTGTACGCCGCATTTATCCACGCACAACTGTTTGTAATGTCATGCCGGAGCAAACTGACTAAGTTC
CTGCTGCTCATTCCCATGATGTTGCTGCCCAAAGCCGTTCTTGCC
(MSRIRVVAARLYAAFIHAQLFVMS**CRSKLT**KFLLLIPMMLLPKAVLA)

102.
GCCGCGACGAGCGCCAGCGTAACGTTAAGCGACGCGCAAGCGAAGCTCATGCAGCCAGGCGTCAGCGA
CATTAATATGGAACGCGTCGAAGCATTAAAAACGGCTATCCGTAACGGTGAGTTAAAAATGGATACGG
GAAAAATAGCAGACTCGCTCATTCGCGAGGCGCAGAGCTACTTACAGAGTAAATAAGCGTATGACTCG
TTTGTCAGAAATACTT
(NO ORF)

103.
GGCGAGGGCAAAGCGGATAGCGACGGTAAATTTAGTATTGATCTGACAGCGCCACAGATTAGCGGCGA
ACAACTTACCGTGACCGCGACTGACGATGCCGGCAATACCGGCCCATCGGCAACCATTGATGCGCCCA
ACATTCCTCTCCCCGATACACCGGTTATCACCGCCGCTATCGATGATGCCGCTCCCCTCACCGGCACG
CTGAGCAATAATCAGTTTACGAACGACAATACCCCCACTTTGGAGGGCACCGGCAGCGCAGGCACAGT
CATCCATATTTACGCCAATGGTCAGGAAATAGGCTCAACAACGGTTGATACCAGCGGAAACTGGCATT
TTGCCATTACCAGCGCGCTAGCGGATGGGGAAAATCATTTCACC
(GEGKADSDGKFSIDLTAPQISGEQLTVTA**TDDAGN**TGPSATIDAPNIPLPDTPVITAAIDDAAPLTG
TLSNNQFTNDNTPTLEGTGSAGTVIHIYANGQEIGSTTVDTSGNWHFAITSALADGENHFT)

104.
GTGGAATAGCGGTAACCCAGCAATTGAAAATTAGTCCCGTAATTATTCAGCGATTTGGCGTACAGAAA
ACGCAACGATTGCCCCTGATGGGTGCTGTCATCTGCCAGTTGGC
(GIAVTQQLKISPVIIQRF**GVQKTQ**RLPLMGAVICQL)

105.
AGCGTCGCCGACGAGCTGATGCGTCTGATGGAAGGTCAGCACGCGGTGAAACTGACCGCCGCTCAGGC
CGAACAGCTCCAGCCGGTGCTGCTGAAGAATATCGATGAACGCGGCAAAGGCACCGTCAGCCGTGACT
GGGTCGGTCGTGACGCGGGCAAGATCGCCGCAGCCATCGGCCTGAACGTCCCGGATCAAACGCGCCTG
CTGTTTGTGGAAACGCCCGCCAACCATCCGTTTGCCGTTACTGAAATGATGATGCCGGTACTGCCGGT
GGTGCGGGTCGCCAACGTCGAAGAAGCCATCGCCCTGGCGGTTCAGCTTGAAGGCGGTTGCCACCATA
CGGCGGCGATGCACTCGCGCAATATCGACAACATGAACCAGATGGCGAACGCCATCGACACCAGCATT
TTCGTCAAAAACGGGCCGTGCATTGCCGGGCTTGGATTGGGCGGAGAAGGCTGGACCACCATGACTAT
CACTACGCCAACCGGCGAAGGGGTGACCAGC
(SVADELMRLMEGQHAVKLTAAQAEQLQPVLLKNIDE**RGKGTV**SRDWVGRDAGKIAAAIGLNVPDQTR
LLFVETPANHPFAVTEMMMPVLPVVRVANVEEAIALAVQLEGGCHHTAAMHSRNIDNMNQMANAIDTS
IFVKNGPCIAGLGLGGEGWTTMTITTPTGEGVTS)

106. DELETION MUTANT

107.
AGTTTTGAGAGAGCCTTGCCGGGTTTTCATCGCAACCTTCCGCTAATTATTTGAGAAGGGTAAATAAT
ATTGAAATCTTAACAGTG
(NO ORF)

108.
ATTCCCGGGATGAACAATATCACCGTGATCTTGCGGGAACCGCAAACCCTGGCGCTGGATGCGATTGA
GCGTCTGCAGCGCTGGTGGGAAGAGAGC
(IPGMNNITVI**LREPQT**LALDAIERLQRWWEES)

109. (SEQUENCE NOT AVAILABLE)

110.
ACCCTTTTGACCTTTTACTCCCTGCAGCAATGGCCAAAGTGGCTGAAGAAGCAGGCGTCTATAAAGCA
ACGAAACATCCGCTTAAGACT
(PFDLLLPAAMAKVAEEAGVYK**ATKHPL**KT)

111.
GATGACGACCTCATCAATGGATTTATGGCACATCAGGCAAAAGGCCTTTTTTTTTATGCGCCTGCAATG
TTGAATCCCACGCCGCTGATCCGCCTTCTTTATTCACTTTTTCTCTTATCGCCGCCAGCCGATCTATT
TCATAATAAGCGCTCAGGCAGCCACAATGGGGGCATAATACGGGGCCATTATTTATTTTCATACTATT
TCCTGCTGTGGTGAGGCTATCGGGTAAAGTAACAATCCTACCGTAACGTAGCAATCTCTATACCATAA
ATGAAACCTCAATAGGTGTATCCCTATTCTGAAAAGGGGATAACAACCAGGATTCAACGCGTGATCCA
CAGCGTAGGCCAGGCATCCGGCCCGTGCCAGCTATC
(NO ORF)

112.
GCAGATGCCGGGATAACTCATCACAGAAATAATCGATTTATAATAACGCAAATAATTCCTGATGCCTT
ACTGAGCTCCGGCTAATGCGGGGTGGATAAGCTGGAATACAGACCCGGTAATACACAGCGATTATATT
AACAAGCAATGTAATAAAGGAGTTTTTTATGTTCATGTTTCTGCCATTCTTGCTTGCACTGAGCGTGG
CAATGGGGGCC
(NO ORF)

113.
GGTCGATCTCCTCTTCGCCCTGCTGGTGCCCGCCGATCAAACTAAAACGCATCTGCACACGCTGTCGC
TG
(VDLLFALLV**PADQTK**THLHTLSL)

114.
GGTATCCGCCGTCCAGAATCAACTCACAAAACAACCTCTCCACAGCCAGGAGTTACTTGATCCCTTGC
GGGCGATGCTGGCAAAAACGCTGGCGGCGCTGACACCGGGGAAACTCAAGTACAGC
(VSAVQNQL**TKQPLH**SQELLDPLRAMLAKTLAALTPGKLKYS)

115.
CTGAATTAAGGAGCATTCCATGTCTGCTTTTACCCCGGCAAGTGAAGTCTTGCTGCGCCACAGTGATG
ATTTCGAACAAAGCCGTATTCTTTTTGCCGGAGATTTGCAGGATGACCTGCCCGCGCGCTTTGAATGC
GCCGCCAGCCGCGCGCATACGCAACAGTTTCACCACTGGCAGGTATTAAGCCGCCAGATGGGCGATAA
CGTCCGTTTTAGCCTGGTCGCGCAAGCCAGCGATGTCGCTGACTGCGATACGCTGATCTACTACTGGC
CGAAAAATAAACCCGAAGCGCAGTTCCAGTTGATGAATATTTTGTCGCTGATGCCGTCG
(IKEHSMSAFTPASEVLLRHSDDFEQSRILFAGDLQDDLPARFECAASRAHTQQFHHWQVLSR**QMGDN**
VRFSLVAQASDVADCDTLIYYWPKNKPEAQFQLMNILSLMPS)

116.
CATGTTATCTGGGACAGCAACAGCAAACTGATGGATAAGTCTGACGCCCGTCACTGCACAGTTATCCA
CTCAATGAGCCAGAATGGTTGGGAGGATTTCGCAGAAAAATACGACCTCGATGCGGATGATATTCCAT
CATTCCAGAACCCCAACGATTGGGTATTTCCATGGCTGACGCAGGACACAATTCAG
(HVIWDSNSKLMDKSDARHCTVIHSMSQNGWEDFAEKYDLDADDIPS**FQNPND**WVFPWLTQDTIQ)

117.
CAATTAAACGTAAACCGTTCACACGGCAAGCCCCAGATATATAAATCTGATTCGCCTGGAAATCCTTA
GTATTGGTGTCAATTATGGCTGGGAGATTCACTATGCAGTTTTCAACAACCCCAACGCTGGAAGGACA
AAGCATCGTG
(QLNVNRSHGKPQIYKSDSPGNPWYWCQLWLGDSLCSFQQ**PQRWKD**KAS)

118.
GAAAGTACGCGACAAAGGTTTGTCGGAAGAGGAGTTTACTGCGCTGGTGGCGCAGAAAAATCTCGAAT
TGCAAAAGCTGTTCGCGACCTACGCGCGTACCGATACTGACATTTTGACT
(KV**RDKGLS**EEEFTALVAQKNLELQKLFATYARTDTDILT)

119.
CACGTGATTAAGCGGGAAAACTATTTCGTTAGTTTGCGCTTCTGACTACAATAGCCGCCATTTCGCTT
CTATTGGATAAGAACGACTGTATGCGCCAGCAACCTCACTATCTCGAATTGTTAAGTCCGGCCCGTGA
CGCCGCAATTGCTCGCGAAGCGATTTTGCATGGCGCAGATGCCGTCTACATCGGCGGACCCGGTTTTG
GCGCACGTCATAACGCCAGTAACAGTCTGCGCGATATCGCCGATCTG
(MRQQPHYLELLSPARDAAIAREAILHGADAVYIGGPGFGARHN**ASNSLR**DIADL)

120.
ATCCGCAAAAGTGCGTCCCGCTGGCCGATCATCAAACGCTCGCCCCTGCTGACGTTCAGGCGTTGGTC
GGTGAAGGGCAGACCCTGGTAATG
(**PQKCVP**LADHQTLAPADVQALVGEGQTLVM)

121.
TGGGTGGAACGCCATCAGCCGTTGCTGGAAAAACACGTTCTTTATGCAACCGGCACCACGGGGAATCT
GATCCAGCGCGCAACCGGTATGGACGTTAATGCGATGCTGAGCGGCCCGATGGGCGGCGACCAGCAGG
TTGGCGCACTCATTTCAGAAGGGAAAATCGACGTGTTGATTTTCTTCTGGGAC
(WVERHQPLLEKHVLYATGTTGNLIQRATGMDVNAMLSGP**MGGDQQ**VGALISEGKIDVLIFFWD)

122.
CAGTACGGCCAAGGCTGCAACTAACATGGTTGAAAATGCAATGACTCGTGATTTGCGCAATGCTGTCG
GTAAATCACTAGGGCCACAAGATGCAGCAAAATATCTCAAATCCAACTCAGACTTCGCAAACATTTAC
AATAAGGTTCTGAATAAGCGCATCTCTAATACGCTAAATAAAGCCAGAAGCGAATACACACCCGAGCT
TATTAACACCGTTGTTTTCAGTCGCAAACCGTCAGATATAAAGCGC
(STAKAATNMVENAMTRDLRNAVGKSLGPQDAAKYLKSNSDFANIYNKVLNKRISNTLNKARSEYTPE
LINTVVFS**RKPSDI**KR)

123.
GAGTAAATAAGCGTATGACTCGTTTGTCAGAAATACTTGACCAGATGACCACCGTCCTGAATGACCTG
AAGACGGTGATGGACGCCGAGCAACAACAGCTTTCCGTAGGCCAGATTAACGGC
(VNK**RMTRLS**EILDQMTTVLNDLKTVMDAEQQQLSVGQING)

124.
GAAATAACATGCCGCCGTCAGCCGCCTCGAACGCGCCCGGTCTGTCGCTCTGCGCGCCGGTAAACGCG
CCCTTC
(**NNMPPS**AASNAPGLSLCAPVNAPF)

125.
GTGATGGACGTGGCGAACGCCGTCCTGGATGGCACGGATGCGGTTATGCTGTCTGCCGAAACCGCAGC
CGGTCAGTATCCTTCTGAAACCGTTGCCGCAATGGCGCGCGTCTGCCTGGGCGCAGAAAAAATCCCCA
GCATCAATGTGTCTAAACACCGTCTCGAC
(VMDVANAVLDGTDAVMLSAETAAG**QYPSET**VAAMARVCLGAEKIPSINVSKHRLD)

126.
GTATATTGATGAACGCTACGCCGCGCCGTTGACGCGCGAATCGGTAGCACAGGCGTTTTATATCTCGC
CAAATTACCTCTCGCACCTGTTCCAGAAAACGGGGGCCATC
(**YIDERY**AAPLTRESVAQAFYISPNYLSHLFQKTGAI)

127.
GGACATCACTTCTGATAATCAAGCGTTTAATAATATATTAGAGGGTATTGATATAATAGAATGTGAGA
ATTTATTAAAAGAAATGAATGTGCAAAAAATACCTGAATCCTCTCTTTTTACAAACATTAAAGAAGCT
TTACAGGCAGAAGTTTTCAATAGTACTGTAGAAGATGACTTTGAGAGTTTTATTTCTTACGAATTACA
AAACCATGGACCACTGATGTTGATCAGGCCTTCACTTGGCTCGGAATGT
(D**ITSDNQ**AFNNILEGIDIIECENLLKEMNVQKIPESSLFTNIKEALQAEVFNSTVEDDFESFISYEL
QNHGPLMLIRPSLGSEC)

128. (SEQUENCE NOT AVAILABLE)

129.
CTCTCACTGACCTGACTCACATACGCCAACTGGGGCTGAGCCTTATTCTCTCGTTCTGAAGGACAGAA
GCACAAGCGCATGATTTA
(NO ORF)

130.
AGATTCGGGACAATGACAATGAAAAAAGTTCTGATTGCCGCGCTAATTGCAGGCTTTAGCCTTTCCGC
TACAGCAGCCCAGACCATTCGTTTTGCGACCGAAGCGTCCTATCCGCCGTTCGAATCGATGGATGCTA
ATAACAAGATTGTCGGCTTTGACGTCGACCTGGCCAACGCGCTATGTAAAGAGATCGACGCCTCCTGT
ACCTTTACCAATCAGGCGTTCGACAGCCTGATCCCCAGCCTGAAATTCCGCCGCTTCGACGCTGTAAT
GGCG
(RFGTMTMKKVLIAALIAGFSLSATAAQTIRFATEASYPPFE**SMDANN**KIVGFDVDLANALCKEIDAS
CTFTNQAFDSLIPSLKFRRFDAVMA)

131. SEQUENCE NOT OBTAINED

132.
ATAAATTCGACCGTCTTCGCATCTTCATCACGCGGGCCGAAATTCCATCCTTCAGAAAACTTAGCACC
TTCTGTATATAAGCGTTGCGCCACCACAATGTAACCAGAAAGAGGCTCCAGTACATGCTGCCAGGGAC
GGATAGAATATGGGTTTC
(NO ORF)

133.
CAGACGACAATAGTGTAACTGCCCTCTTTCTTGGACTGTTCCGGTATCCCAGGAGAAAGTACTTTATC
AGTGTTCATCTCTCTTATGATGCCCGTGAACCCAAATGCGTCGCGGAACCTACTTTTACCTTCTTCTG
ACAAGAGCAAGAGTCTGGCACGATGAATACCTTCCATGACCTCCCGGAGCGCGCGGCGCTGCTCATCC
ACGATATGCAGGATTACTTTGTCAGCTTTTGGGGCCGCAACTGCCCAATGATGGACCAGGTGATTGCT
AATATC
(NO ORF)

134.
GAATGCGGGCTACCTTGCTCAGTCAGGCAATACTAAAAGCTCTTCCTTAACGGCTGACACGGCGATGA
CCTGGTATGGACAACGTACC
(NAGYLAQS**GNTKSS**SLTADTAMTWYGQRT)

135.
CCCGGGTCGCTGCCGGTGAATCTGGCGCAACTGAAATCAATGCGCCGTATTCGCGAGTTTGACGACCT
GATCACCGCGAAAATTCACGGCTTTGCCGACGCCATCGACTACTATCGTCAGTGTAGCGCCATGCCGT
TGCTTAACCAGATTGCTAAACCGACGCTGATTATTCACGCTAAAGACGATCCGTTTATGGATCATCAT
GTGATCCCTAAAGCGGAAGATCTGCCCCCGCAGGTGGAGTATCAGCTGACTGAGCATGGCGGACACGT
AGGATTTATCGGCGGTACGCCGTTGCGCCCTGAAATGTGGCTGGAACGCCGAATTCCTGACTGG
(PGSLPVNLAQLKSMRRIREFDDLITAKIHGFADAIDYYRQCSAMPLLNQIAKPTLIIHAKDDPFMDH
HVIPKAEDLPPQVEYQLTEHGGHVGFIGGTPLRPEMWLE**RRIPDW**)

136.
TGGCCGATGATCAGTTTGTTGCTACTGGGGCTGGGTCGCGTGATTTTAGGGATTGGGCAAAGTTTTGC
CGGC
(WPMISLLLLGLGRVILGI**GQSFAG**)

137.
GAGATGATGGCCTCTCTGCCTGCGCAGTGGCGCGATATTGATGTGCTGGTCAATAACGCCGGTCTGGC
GCTGGGTCTTGAACCGGCGCATAAAGCCAGCGTAGAAGACTGGGAAACTATGATCGATACTAATAATA
AAGGCTTAATTTATATGACCCGCGCCGTGCTGCCAGGAATGGTCGAACGTAATCGCGGTCATATCATT
AACATCGGTTCAAC
(EMMASLPAQWRDIDVLVNNAGLALGLEPAHKASVEDWETMI**DTNNKG**LIYMTRAVLPGMVERNRGHI
INIGS)

138.
TTGATAATGTGGAACAGGCACTGAGACATTCATCTGATGGCCTGATTCTCCGGCTACTGGATATACAC
GAGGTCGGGCGTGAAATACGTCGTCAGACAGAACGGCACAGCACACCGCGTAACTGGCAACCCCACCT
GCCTGAAGATGCCCAGCCAGCGGGATATCGCTGGACTGACGATGAATCCGTACTACATGCTCCTTCAT
TGCATTTGCAGTTATTCAACACTCAGGTGACCACACAGGGCAACCTG
(DNVEQALRHSSDGLILRLLDIHEVGREIRRQTERHSTPRNWQPHLPEDAQPAGYR**WTDDES**VLHAPS
LHLQLFNTQVTTQGNL)

139.
AGAGCCGTTGTTTCCACTGTGGAACATGGCTGTAGTCACGTTGCACCCCGACGCTCCCCACCTCACCG
CC
(EPLFPLWNMAVVT**LHPDAP**HLTA)

140. (SEQUENCE NOT AVAILABLE)

141.
CCCAGAACCGGCAGCGGGCTGGATTCCTGCAGTTTAGCAGGATCGATTTTGATCACCTGCGCTTTGGA
AGAGTCGTCAAAGATCTCCGACAGATCCGGGCGAGTACGGCCTTGT
(PRTGSGLDSCSLAGSILITCALEESSKI**SDRSGR**VRPC)

142.
GCAGTGATAGAAAACCGGAATGAACTGAGGGTTTATGCGTACCGCTCTGAATATTTTAAACTTTGTAC
TGGGCGGCTTTGCCACTACGCTGGCCTGG
(**MRTALN**ILNFVLGGFATTLAW)

143.
TAACTCATTTTTTCATTAAAATAAGTAAAATCATAAAGATGCAAACATTCACATGACGCCAGACAAGA
TTAAATCTTCTCCTGACTGCGTTCTCTCCTTCCAAAAAGTCAAAAAATAGCCCATTGCCTTCTTGAGA
AAATAGTGTCAGGATTAATCAAAACAAATCACGTTTTGAACCATTTTGAACAAACATGAAGGTAAATG
CGATGA
(NO ORF)

144. (SEQUENCE NOT AVAILABLE)

145. (SEQUENCE NOT AVAILABLE)

146. SEQUENCE NOT OBTAINED

147.
CCATAATTAAAGGAACAACGATGCGACATACCGTGATATTTGCCTCAGCGTTTGCCACCCTTGTCACC
GCCAGCGCTTTCGCTGCCGACCTGCCTGGCAAAGGCATTACCGTCCAACCTATCC
(IIKGTTMRHTVIFASAFATLVTASAFAAD**LPGKGI**TVQPI)

148.
CGCGGACATCCCGAATAAGCCGCTGTTTCTCCAGAACGTTGGTTTAGTAGACGTTCTTTTTAAAGGTG
ACGGGCGATTCCTCGCGGGAACGTTTGTCAGTGACGCAATTGACCGAACATCAATCGGCGCGCGT
(ADIPNKPLFLQNVGLVDVL**FKGDGR**FLAGTFVSDAIDRTSIGAR)

149. GGCGGGGAAATAGCTAAATAATGGCGGTAACGGTTCGCTGAAAAAGAGCGGCTGCCAGTATTC
(NO ORF)

150.
CGAATGATATTGTATAATTTTTATTTTGTATAATACCCCCAAAAGCATTCGTATAAATTATATCTATT
TCACTGCGAATTATTTCATTAATTATTGAATTAAACGGTAACATCTCTTTTTAGGTCTTTCCTGACAA
GGCAGAAATAACGTTTTAACGTCAACTCGCTGATTATTTACGTGGAATAC
(NO ORF)

151.
AACGTCTTTAAAACGTTTCTGTTTATCCCGGCAATTCTCATTGGCGGCCATCTGGCGGGGGCGATTGG
CGTGACGCTG
(**NVFKTF**LFIPAILIGGHLAGAIGVTL)

152.
GCTATATTGAGCCACAGCTACCTGACGTCGCGACATTGAAAGATGTGCGTTTGCAAATTCCGATGCAG
GTTTACAGCGCGGATGGCGAACTTATTCGCG
(YIEPQLPDVATLKDVRLQIPMQVY**SADGEL**IR)

153. CGCCGCTATCGTCAGGGTATTGTCATCAAACTGGATAACGTTTTCCGGG
(AAIVRVLS**SNWITF**SG)

154. (SEQUENCE NOT AVAILABLE)

155. (SEQUENCE NOT AVAILABLE)

156.
GGAGGGACAACGGTGATCGATCCCGTGCTTGAATACCGCCTGTCACAGGTTCAGAGCCGCATTAGCGA
AGAGCGCTTTCTCA
(GGTTVIDPVLEYRLSQVQS**RISEER**FL)

157. SEQUENCE NOT OBTAINED

158.
CGGTTCTGAACCGAAAAACGATCGTCCGTCCTACATCTGATACCCACGAGGCTGATTCATGAGATCGA
AAAGATTTGAAGCACTGGCGAAACGCCCTGTGAATCAGGACGGCTTTGTTAAGGAGTGGATCGAAGAA
GGCTTTATCGCGATGGAAAGCCCGAACGACCCAAAACCGTCGATAAAAATCGTTAACGGCGCGGTAAC
CGAGCTGGACGGAAAACCGGTTAGCGAATTCGACCTGATCGACCACTTTATCGCCCGCTACGGCATCA
ACCTGAACCGCGCCGAAGAAGTGATGGCGATGGATTCGGTCAAGCTG
(NO ORF)

159. (SEQUENCE NOT AVAILABLE)

160.
ACCGGTGGTGGCGGAAAAAGTATTCCCTGGCCCGTTTGACGACTGATTCATCCTGCGGCATGTCTGTC
TC
(RW**WRKKYS**LARLTTDSSCGMSV)

161.
CGTGCCTGACCCGCCTAACAGGCTAATTCCCCCCAATACGCAGGCCGCGATGGCTTTCATTTCCAGCC
CCGTGCCGGTT
(V**PDPPNR**LIPPNTQAAMAFISSPVPV)

162.
GTTAAACGCCTCATCTTCCTTCAGGTAACTTTTAATGTTTTCTGCCCGGGCAGCCATTTCCGGATCAT
GTTTAAGT
(VKRLIFLQVTFNVF**CPGSHF**RIMFK)

163.
GCTTGGCATCGTCGAGAAAAGTGCCGTCCATATCTGTAACGACAACGTTAACGGTCATACTTAAGCTC
CTGGCTGTAACTGCGAC
(L**ASSRKV**PSISVTTTLTVILKLLAVTA)

164. SEQUENCE NOT OBTAINED

165. SEQUENCE NOT OBTAINED

166. SEQUENCE NOT OBTAINED

167. SEQUENCE NOT OBTAINED

168. SEQUENCE NOT OBTAINED

169.
GATCAGACGGTGATGGTGTGGCTGGCGTGGCCGTTCGATCCGGTTTTAAAATTTGAAGTCTGGCGCTA
CTTCACCCATATCTTTATGCACTTCTCACTGATGCATATTCTTTTTAACCTGCTGTGGTGGTGGTATC
TCGGCGGGGCGGTA
(DQTVMVWLA**WPFDPV**LKFEVWRYFTHIFMHFSLMHILFNLLWWWYLGGAV)

170. DELETION MUTANT

171.
CCATACTTTTCAGGCGACCGGCCCACAGATCGCTGATGGGCAGCATCAGATCGCCACGACGAAACAAC
TGCTGATACTCCGGCGTGTAATGACTGAGCACCTCACGACTAGAGATATC
(HTFQATGPQIADGQHQIATTKQLLILRRVMTEHLTTRDI)

172. (SEQUENCE NOT AVAILABLE)

173. TAGGTATAAATGATGAGGTATAAGGAACAGGAGTCTGTAATGAAA
(NO ORF)

174.
CCGGTAGAAAATCCCGGCATCGCCGCACGTTTCACCCGCGATCCTGACGCGGTAGTAGCGAAAGTACG
TCATGCTTTTGCAAGCGATAAACCCAGGCTGCGCTATCCCGTTACGCTGGTCACCTGGGCGGTGATGG
CGCTGAAACGCATATTGCCGGGCCGGATGCTGGATAAAATTTTACAAAGCTGAGTTGAAGCGCGCCCG
TCAGCCCCCATGTAAACGTGACACAGACAAAAGAGAGCGACGCCATGTCCGTACAGAACATTGTCAAT
ATTAATGAATCCAACCTGCAGCAGACCTTAGAGCAGTCCATGACCACGCCGGTACTG
(FOUR STOP CODONS FOLLOW THE CDS)

175.
GATATAAATAAAATTGCTGTGCGGGAATATAGCGGGTCAGCAACGACACTAACGCCAACAAGGCCGTG
ATTAAAATAGCATACACCGGCACGCCGCCGCCGTTCGTTTTACCAAAGCAGGCTGGCGCAAAGCGATC
GCCCGCCATAGACCACAGCATACGCGAACTG
(NO ORF)

176.
GACAGATGGACCCAGGGCTGGTCTATAACGCCGTTCGGGATGGGCTGGTTGACGCCGGGCTGGTCTAT
ACCACCGACGGACGGGTGAAAGGGTTTGATCTGAAAGTGCTGGAAGATGATAAAGGCTTCTTTCCAAG
TTACGCTGTCACGCCCGTGGTGCGTAAAGAGGTGCTGGAAGCCAATCCTGGCCTTGATGACGCCTTAA
ACACCCTTTCCGGCCTGCTCAATAACGATGTGATATCGACCCTAAACGCTCAGGTCGATATCGAGCAT
CGCACGCCGCAACAGGTAGCCCATCAATTTTTGCAGGACAAAGGTCTGCTGTAAGGAGCGACTATGGA
TACGATACATTATATGCTGGATAACGCAGGCTATCTC
(QMDPGLVYNAVRDGLVDAGLVYTTDGRVKGFDLKVLEDDKGFFPSYAVTPVVRKEVLEANPGLDDAL
NTLSGLLNNDVISTLNAQVDI**EHRTPQ**QVAHQFLQDKGLL)

177. SEQUENCE NOT OBTAINED

178. SEQUENCE NOT OBTAINED

179.
GTGAGCAGCGCCGCGCAGAGCGCCCGGCCATCGAATTAGGTAAACTGGTGCTTGACTGGAACCCGGAA
ACCAAAAAAAGTGGACAGCTACAATGGTAAGTTGATCACCATGTATGCGGATACTTATAAGCCAGATCC
GGTCACGCAGGCCAAAATTGACGAATGGGATAACAAGGTTAAGAAAATTACCGATGAGGTGGTCG
(EQRRAERPAIELGKLVLDWNPETKKVDSYNGKLITMYAD**TYKPDP**VTQAKIDEWDNKVKKITDEVV)

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